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A Study of the Involvement of Endothelins in the Effects of Hypoxia on the Rat Lung

Submitted by Robert M. Smith

For the degree of PhD

of the University of Bath

1997

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**"...when you look long into an abyss,
the abyss also looks into you."**

Friedrich Nietzsche.

To Mum, Dad and George.

Summary

A ventilated, perfused isolated rat lung is described, allowing simultaneous measurement of pulmonary inflation pressure (PIP), pulmonary perfusion pressure (PPP) and lung weight (LW). The model has been demonstrated to be stable for a period of 2hrs under normoxic conditions (20% O_2 / 75% N_2 / 5% CO_2). Exposure of the lung to a systemic hypoxia (95% N_2 / 5% CO_2), both in a single pass and recirculating system, resulted in an increase in both PPP and LW. No significant change in PIP was seen.

The increases in PPP and LW could be blocked by the ET_A receptor antagonist BQ123, the ET_B receptor antagonist BQ788 and the mixed ET_A/ET_B receptor antagonist bosentan, indicating the involvement of both the ET_A and the ET_B receptor in the responses seen. Furthermore, the endothelin converting enzyme inhibitor phosphoramidon, and agents which disrupted the function of the cytoskeleton and prevented secretion (colchicine and phalloidin) and inhibited peptide synthesis (cycloheximide) also prevented the increases in PPP and LW induced by hypoxia.

Exposure to hypoxia in a recirculating system results in an elevation of perfusate ET-1 levels. However, tissue ET-1 levels do not alter significantly, indicating synthesis and release of ET-1 in response to hypoxia.

In addition to this, prepro ET-1 mRNA levels increase in whole lung homogenate following hypoxic perfusion, suggesting an upregulation of ET-1 production.

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Introduction

HYPOXIA AND HYPOXIC PULMONARY VASOCONSTRICTION

Hypoxia is defined as "...a diminished amount of oxygen...". Hypoxia has many effects on tissues, which vary depending upon their location. Hypoxia causes dilation in systemic arteries, but constriction of the pulmonary vasculature (for review see Wadsworth, 1994). This hypoxic pulmonary vasoconstriction (HPV) is an important physiological response to maintain the ventilation/perfusion ratio and facilitate optimal oxygen uptake by the pulmonary circulation (Fishman, 1976). As HPV is a powerful active control mechanism in the pulmonary circulation it has been an area of intense debate and investigation. The first description of a response to hypoxia was noted by Bradford and Dean (1899). Modern research into the phenomenon of HPV was instigated by von Euler and Liljestrand in 1946, when they demonstrated the hypoxic pressor response in a series of elegant experiments in the anaesthetised cat. The vasoconstriction seen in response to hypoxia was not new, but the paper excited considerable interest.

Despite more than four decades of investigation into HPV, the mechanisms responsible for the response are still to be defined. Early research showed that autonomic activity was not essential for the development of HPV, as the response persists in isolated lungs (Hauge, 1968; Fishman, 1976; Szidon and Flint, 1977) and in transplanted human lungs (Robin *et al.,* 1987). HPV is also unaffected

by adrenoceptor blockade (Malik and Kidd, 1973), catecholamine depletion (Goldring *et al.,* 1962), sympathectomy (Fishman, 1961) and depletion of neuropeptides (McCormack *et al.,* 1993). It therefore seems that intrinsic mechanisms within the vessels of the lung are responsible for HPV.

Recent reports have indicated that there are two main components to HPV. There is a rapid transient constriction of about 5 min, which generally falls towards, but does not reach the baseline (phase 1, Jensen *et al.,* 1992), which is commonly seen in pre-contracted preparations, and a slower, more sustained, phase (phase 2) which is endothelial dependent in most species (Jin *et al.,* 1992, Ward and Robertson, 1995). The transient phase 1 hypoxic pulmonary vasoconstriction appears to be due to K^+ channel blockade (Weir and Archer, 1995), but the mechanism(s) underlying the phase 2 response are not clear.

Two main hypotheses for the mechanism(s) responsible for the phase 2 HPV have been proposed. The first is the mediator hypothesis, where endogenous constrictor substances are released, endogenous dilator substances are suppressed, or a combination of the two occur, to elicit the HPV response (see Figure 1.1). The second hypothesis is a direct action of hypoxia upon the pulmonary vascular smooth muscle to elicit contraction (Barnes and Liu, 1995).

Several vasoactive substances have been nominated as candidates for the vasoconstrictor mediator in the lung, including catecholamines (Fishman, 1976); histamine (Hauge, 1968); angiotensin-II (Berkov, 1974); 5-hydroxytryptamine (Fishman, 1976); platelet activating factor (McCormack *et al.,* 1989a); ATP (McCormack *et al.,* 1989b) and prostaglandins (Weir *et al.,* 1976). All of these have proved to be non-essential for HPV, although they may all play a modulatory role in HPV (Barnes and Liu, 1995). Endothelins have also been proposed as a mediator for chronic (phase 2) HPV, but their onset of action is too slow, and duration of action too long, for them to mediate the acute (phase 1) HPV seen in some preparations. Several dilator substances have been proposed to be involved in HPV, including bradykinin (Weir, 1978), prostacyclin (PGI_2) and nitric-oxide (NO). Bradykinin has been discounted as a vasodilator mediator of HPV in the rat, as it has been demonstrated to cause vasoconstriction in the rat lung (Lal *et al.,* 1994). It is interesting to note that in preparations with raised tone bradykinin has been shown to cause vasodilatation (for review see Barnes and Liu, 1995). However controversy still exists over the roles of PGI_2 and NO in HPV.

The failure, to date, to identify a single chemical mediator has promoted the alternative proposal that HPV may be caused by a direct effect of hypoxia on the pulmonary vascular muscle cells. In support of this hypothesis, small pulmonary arteries of the cat and human have been shown to contract in response to hypoxia in vitro (Madden *et al.,*

1985; Hoshino *et al.,* 1988). Cultured isolated pulmonary artery smooth muscle cells also contract in response to hypoxia (Murray *et al.,* 1990). Several mechanisms have been proposed to account for the direct action of hypoxia on smooth muscle cells. One such mechanism is that hypoxia leads to the closure of an oxygen-sensitive potassium channel, leading to smooth muscle depolarisation, Ca^{2+} entry, and contraction (Lopez-Lopez *et al.,* 1989; Osipenko *et al.,* 1995). To date their are several proposed methods for regulation of a potassium channel by hypoxia, including redox-based modulation of potassium channels by the amount of oxygen radicals present. The free radicals present in the lung act to regulate the ratios of reduced to oxidised glutathione and pyridine nucleotides, which in turn contribute to the redox status of the whole cell (Kozlowski, 1995).

A second redox hypothesis suggests that oxygen tension within the cell regulates the production of reactive oxygen species, which control transmembrane Ca^{2+} flux and hence cell contractility through an action on sulfhydryl groups in the calcium channel within the cell membrane (Archer *et al.,* 1986).

A third hypothesis is the membrane-delimited pathway of oxygen regulation of potassium channels. This theory states that molecular oxygen binds to the channel and is responsible for the open state of the channel. Removal of the oxygen during hypoxia results in closure of the channel, depolarisation and contraction (Lopez-Barneo, 1994).

With the onset of hypoxia, the amount of oxygen available for oxidative phosphorylation is reduced, thus reducing the amount of ATP produced. This would lead to the opening of K_{ATP} channels, which would hyperpolarise the cell and prevent contraction. This does not happen, as it has been shown that endothelial cells are capable of maintaining 75-80% of normoxic levels of ATP production and the same degree of protein synthesis (Pinsky *et al.,* 1995).

The energy state hypothesis suggests that HPV is initiated by decreased oxidative phosphorylation (Rounds and McMurtry, 1981). The cytochrome P_{450} hypothesis proposes that cytochrome P_{450} acts as the oxygen sensor, detecting a fall in PO_2 and initiating HPV (Miller and Hales, 1979).

The possible mechanisms responsible for phase 1 and phase 2 HPV have been explored and documented, although the link from the detection of the fall of PO_2 to the regulation of the levels of constrictor / dilator factors remains to be elucidated.

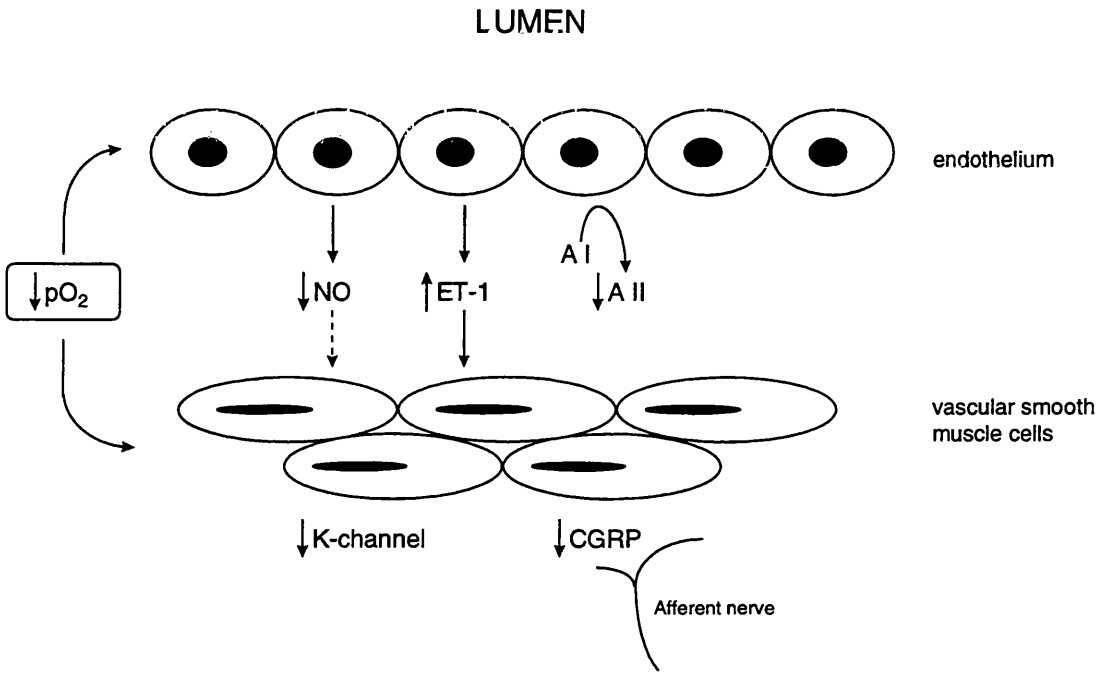


Figure 1.1. Possible mechanisms for hypoxic pulmonary vasoconstriction. Pulmonary vascular tone may increase as a result of release of vasoconstrictor substances, decreased vasodilator substances, or direct action of hypoxia on the vascular smooth muscle cells. Endothelin-1, ET-1; Nitric oxide, NO; Angiotensin-I, A I; Angiotensin-II, A II; Calcitonin gene related peptide; CGRP (from Barnes and Liu, 1995).

MODELS FOR INVESTIGATION OF HPV

Several methods have been used to investigate HPV, ranging from isolated cells to whole animal studies. Cultured isolated pulmonary artery vascular smooth muscle cells have been demonstrated to contract in response to hypoxia (Murray *et al.,* 1990). Pulmonary artery rings from the rat, rabbit, cat, pig, calf, sheep and human have also been demonstrated to contract in response to hypoxia (for review see Wadsworth, 1994). However the response to hypoxia is variable, with responses ranging from rapid contraction (Rodman *et al.,* 1990), a gradual rise in tone (Yuan *et al.,* 1990) or a biphasic response (Bennie *et al.,* 1991; Mathew *et al.,* 1991). This may be due to the type of vessel used, with studies using both large conductance arteries (Yuan *et al.,* 1990) and small resistance arteries (Demiryurek *et al.,* 1991). A second possibility for the difference in the responses seen may be due to the variation in the distribution of various receptor types and ion channels within different regions of the vasculature.

Isolated perfused lungs have also been widely utilised for the study of HPV (Hauge *et al.,* 1966; Hauge, 1968; Berkov, 1974; Marshall and Marshall, 1983; Marshall, 1984). The intact whole lung has the attributes for the investigation of HPV, as the entire vasculature is intact and in contact with other tissue types which may play a role in the development of the responses seen. Studies *in vivo* have mainly been used to examine the effects of long term hypoxia and the

subsequent pulmonary vascular remodelling and right ventricular hypertrophy. In addition to this, the vascular reactivity of pulmonary vessels has been investigated in animals exposed to chronic hypoxia (Emery *et al.*, 1981).

ISOLATED PERFUSED RAT LUNGS

The isolated perfused lung preparation has been reviewed several times in recent years (Mehendale, 1981; Niemeier, 1984; Marshall, 1984; Bhattacharya, 1989). Depending upon the experimental requirements, the lung may be perfused with whole blood, plasma, or a plasma and blood free physiological salt solution (for review see Czartolomna *et al.,* 1991; Chang and Voelkel, 1992; Rodman *et al.,* 1992). Blood perfused lungs closely resemble the *in vivo* state, and are utilised in a whole range of experiments, including the investigation of HPV. However these experiments can be costly, due to the volume of blood required (approximately 30-50 ml) for recirculation in each experiment. This may involve the use of several donor animals in the case of small animals such as rats. In addition to this anti-coagulants have to be used, which may interfere with the responses seen. The use of whole blood as a perfusate may also complicate interpretation of the results, as many substances (for example: eicosanoids and their metabolites, lipid mediators etc...) may bind to plasma proteins and/or be metabolised by cells in the recirculating blood. In single pass experiments, a large volume of perfusate is required, making the use of whole blood impractical.

For studies involving single pass perfusion, the use of a physiological salt solution (PSS) is recommended (Chang and Voelkel, 1992). This technique offers several advantages, as PSS perfusion lacks the

components of the blood which can cause the problems mentioned above. In addition, the composition of the perfusate can be precisely regulated, also the lung can be easily lavaged and microscopically examined.

A number of *in situ* and *in vitro* methods have been developed to perfuse and ventilate lungs (Levey and Gast, 1966; Kroll *et al.,* 1986; Niemeier, 1984; Ryrfeldt *et al.,* 1990; Uhlig and Wolin, 1994). The isolated lung, when perfused *via* the pulmonary artery, has been used to assess the release of substances from the lung in response to administration of a number of substances (Bakhle and Vane, 1974; Said, 1979; Robinson and Hoult, 1980; Bakhle *et al.,* 1985; Peers and Hoult, 1986; Conroy *et al.,* 1992).

Lungs perfused in a retrograde fashion *via* the pulmonary vein have also been utilised to study the actions of various vasoactive compounds, which may act upon the venous circulation (Lal *et al.,* 1994). Isolated lung preparations have also been used to investigate the actions of various compounds upon bronchial muscle tone (for review see Lou, 1993). However the effects of these compounds on both pulmonary vessels, bronchial resistance and permeability within the lung are rarely investigated together (Kroll *et al.,* 1986; Ryrfeldt *et al.,* 1990).

Permeability changes within the vasculature in response to hypoxia are not fully understood. Methods to investigate this phenomenon include using cultured endothelial monolayers (Horgan *et al.,* 1991; Rodman *et al.,* 1992). However the use of a cultured monolayer of endothelial cells obtained from one distinct area of the pulmonary vasculature cannot represent the whole pulmonary circulation which is an important part of vascular reactivity. In addition to this a whole lung can be used, this has an advantage over the use of cultured monolayers as there is an intact pulmonary endothelium and alveolar epithelium in a physiological setting (Czartolomna *et al.,* 1991; Horgan *et al.,* 1991; Seale *et al.,* 1991).

PULMONARY FLUID BALANCE AND OEDEMA FORMATION

Formation of oedema within the lung can be regulated in several ways which are explained below.

The movement of fluid across the capillary endothelium is described by the Starling equation:

$$Q_f = K_f (P_{cap} - P_{is}) - \sigma(\pi_{pl} - \pi_{is})$$

Where Q_f is the net flow of fluid; K_f is the capillary filtration coefficient; P_{cap} is capillary hydrostatic pressure; P_{is} is the hydrostatic pressure of the interstitial fluid; σ is the reflection coefficient; π_{pl} is the colloid osmotic pressure of the plasma and π_{is} is the colloid osmotic pressure of the interstitial fluid.

The equation can be represented schematically (see Figure 1.2), and demonstrates the potential causes of pulmonary oedema, which include: an increase in the permeability of the capillary endothelium; an increase in the capillary hydrostatic pressure; a decrease in the interstitial hydrostatic pressure; an increase in the interstitial colloid osmotic pressure or lymphatic insufficiency (Levitzky, 1982). However in the pulmonary circulation plasma colloid pressure is usually greater than capillary hydrostatic pressure, and so there is little bulk fluid movement out of the capillaries.

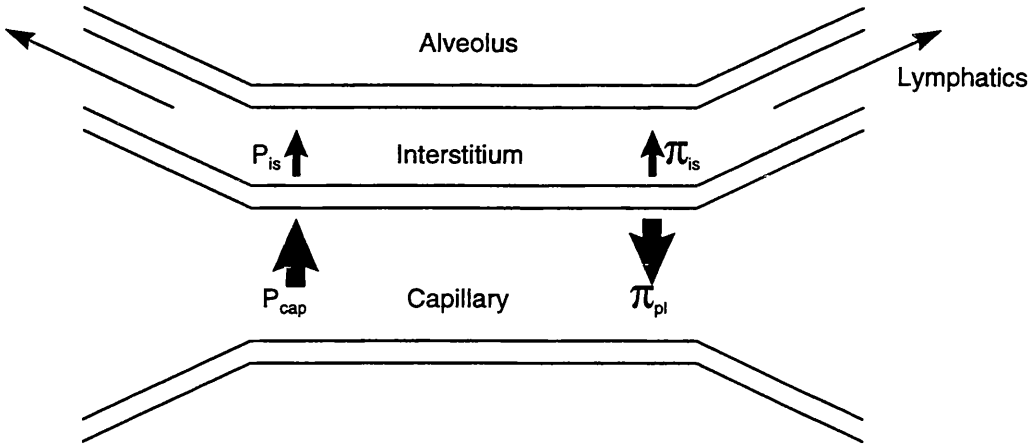


Figure 1.2. Schematic representation of the Starling equation describing the factors affecting liquid movement in and out of the pulmonary capillary. P_{cap} is capillary hydrostatic pressure; P_{is} is the hydrostatic pressure of the interstitial liquid; π_{pl} is the colloid osmotic pressure of the plasma and π_{is} is the colloid osmotic pressure of the interstitial fluid (modified from Levitzky, 1982).

The Starling equation states the laws governing fluid movement across a membrane, but other additional pathways for water and solute movement have been suggested (Staub, 1974). These include movement across cell membranes; movement through intercellular junctions and non-selective leak. Pores crossing cell membranes are water filled channels 4-5 Å radius and only allow water to cross the membrane. The major site of ultrafiltration and protein flow is probably *via* the intercellular junctions (> 1000 Å radius; Staub, 1974).

ENDOTHELINS

Endothelins are potent vasoactive peptides, first described by Hickey *et al.,* (1985). This endothelial-derived constricting factor (EDCF) was then isolated from the culture medium of bovine aortic endothelial cells, purified, sequenced and cloned and renamed endothelin (ET) by Yanagisawa *et al.,* (1988).

It was subsequently demonstrated that this ET was one of a family of 21 amino acid vasoactive peptides, designated ET-1, ET-2 and ET-3, which have been shown to be encoded by three distinct genes. The ETs all contain four cysteine residues (positions 1,3,11 and 15), which participate in disulphide bond formation (Cys^{1}-Cys^{11}; Cys^{3}-Cys^{15}; Inoue *et al.,* 1989). The peptides also have the same amino acids at positions 8, 10, 20 and 21. ET-1 is the isoform originally identified from bovine aortic endothelial cells (originally termed porcine endothelin). ET-2 differs by two amino acids from ET-1, and from ET-3 by six amino acids (see Figure 1.3). In addition to these peptides, a fourth peptide has been discovered following genomic cloning in mice (Saida *et al.,* 1989), and termed vasoactive intestinal contracting factor (VIC) or endothelin-β. This has subsequently been found to be the murine and rat homolog of human ET-2 (Fabbrini *et al.,* 1991).

The three ET isopeptides share a remarkable resemblance, both in structure and biological activity, to the sarafotoxins (Sx), a family of

peptides isolated from the venom of the snake *Atractaspis engaddenis,* suggesting a common evolutionary origin (Sokolovsky, 1992).

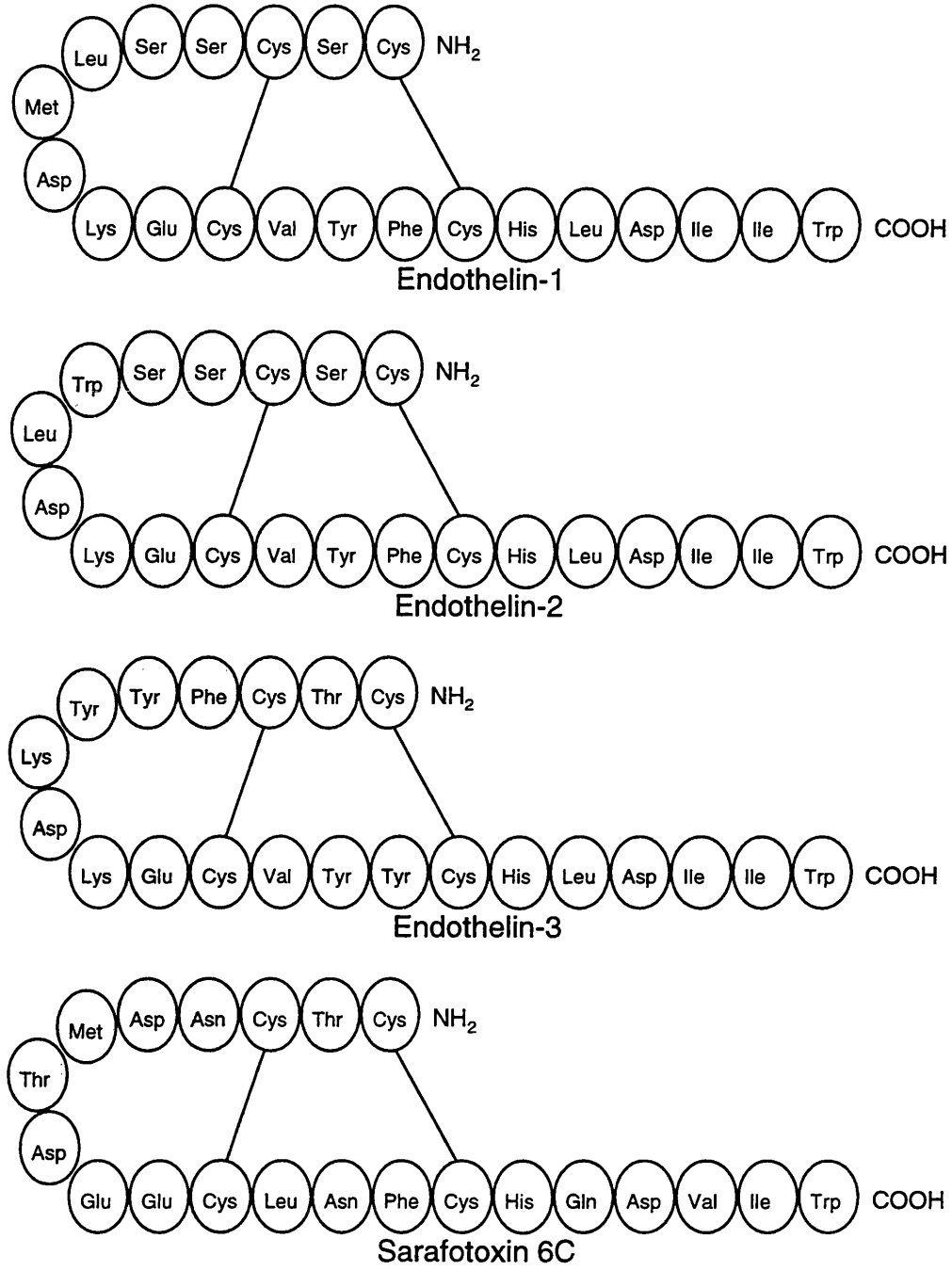


Figure 1.3. Structures of the Endothelins and Sarafotoxin 6C. Shaded amino acids indicate differences from endothelin-1.

ENDOTHELIN BIOSYNTHESIS

The biosynthetic pathway for ETs follows a similar pattern to other peptide hormones and neurotransmitters. ETs are produced by the processing of precursor peptides. These peptides are termed preproendothelins -1,-2 and -3, which consist of approximately 200 amino acids. The preproendothelins undergo cleavage to produce 38 or 39 amino acid peptides known as big ETs. The big ET is then cleaved between positions Trp^{21} and Val^{22} by a specific endopeptidase known as endothelin converting enzyme (ECE; see Figure 1.4).

The chemical nature of ECE still remains a mystery, although two distinct forms have been separated. The first, termed M1 or ECE-1, is a membrane bound enzyme sensitive to phosphoramidon (IC_{50} = approximately 1μM), although not to thiorphan or bacitracin, and shows a pH optimum of 7.0. This ECE is associated with the plasma membrane and appears to convert big ET-1 to ET-1 more efficiently than big ET-2 or big ET-3 (Xu *et al.,* 1994). The other enzyme activity is termed M2, or ECE-2 and is reported to be associated with the Golgi apparatus, and shows an increased sensitivity to phosphoramidon (IC_{50} = approximately 0.3nM), and could also be inhibited by thiorphan and bacitracin, and has a lower pH optimum of 5.5. This enzyme cleaved both big ET-1 and big ET-2, but not big ET-3 (Emoto and Yanagisawa, 1995). It has been proposed that the M1/ECE-1 form of the enzyme represents the previously described vascular endothelial

ECE and is responsible for both intra-cellular and extra-cellular conversion of big ETs to the mature peptides, and that M2/ECE-2 form is the neutral endopeptidase EC 3.4.24.11 that is found in lung, kidney and may be the major ECE in neurones, glia and certain neuroendocrine cells (Goto *et al.,* 1996), and is thought to be responsible for the intracellular conversion of big ETs. M1/ECE-1 is thought to be more similar to the physiologically relevant ECE (Sawamura *et al.,* 1993).

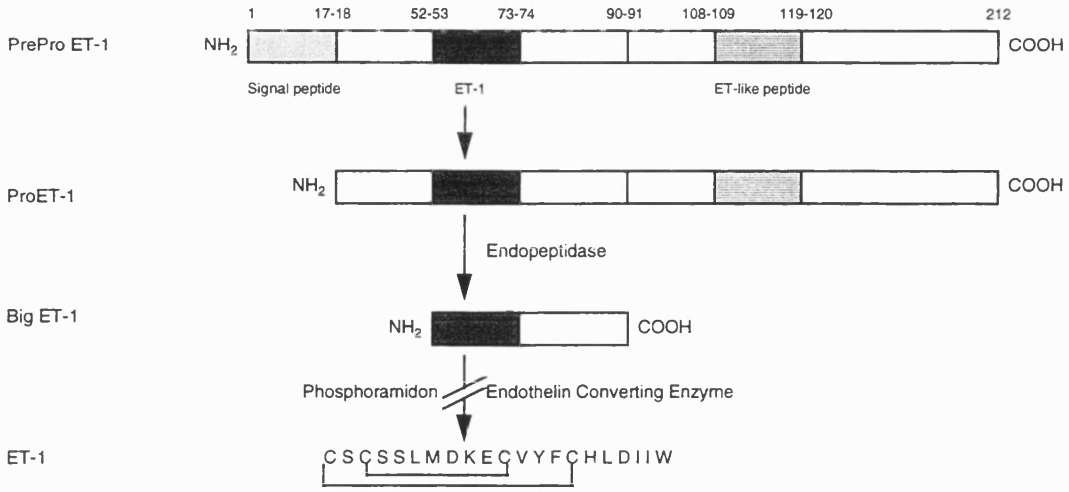


Figure 1.4. Schematic representation of the synthesis of human ET-1

ENDOTHELIN RECEPTORS

The endothelin family of peptides have been shown to exert a wide variety of biological actions, which are thought to be mediated through specific membrane receptors. Two endothelin receptors had been cloned and reported by the end of 1990 (Arai *et al.,* 1990; Sakurai *et al.,* 1990). The first was isolated from a bovine cDNA library, and upon expression in *Xenopus* oocytes showed a greater affinity for ET-1 and ET-2 over ET-3. This was termed the ET_A receptor. The second was isolated from a rat cDNA library and showed equal affinity for ET-1, ET-2 and ET-3. This was termed the ET_B receptor. Following the cloning of the ET_A and ET_B receptors from non-human cDNA libraries, human forms of the receptors have been identified, and the rank order of potency of the ET isoforms confirmed, with ET-1 being approximately 1000 fold more potent than ET-3 on the ET_A receptor (Hosoda *et al.,* 1991) and the lack of selectivity of the ET isoforms for the ET_B receptor (Nakamuta *et al.,* 1991; Sakamoto *et al.,* 1991).

Functional studies however have suggested the existence of more than two receptor subtypes. The most widely proposed of these is the ET_C receptor, which has been cloned from *Xenopus leavis* dermal melanophores. This receptor reportedly shows selectivity for ET-3 over ET-1. This receptor has been shown to have approximately 50% amino acid homology with the ET_A and ET_B receptors (Karne *et al.,* 1993) In addition to the ET_A, ET_B and ET_C receptors a fourth receptor,

the ET_{AX} receptor has been cloned from *Xenopus* heart, and has been shown to share 74%, 60% and 51% amino acid homogeneity with human ET_A and ET_B receptors, and the *Xenopus* ET_C receptor, respectively (Kumar *et al.* 1994).

In addition to the main types of receptors mentioned above, further divisions of the ET_A and ET_B receptors have been made. ET_A receptors have been subdivided into ET_{A1} and ET_{A2} receptors, where the former are BQ123 sensitive, and the later are BQ123 insensitive (Sudjarwo *et al.,* 1994; Yoneyama *et al.,* 1995). However, selective antagonists to ET_{A1} and ET_{A2} are not yet available, and so identification of these receptors is solely made upon the selective antagonism of ET-1 induced responses, either in preparations more sensitive to ET-1 than ET-3, or where selective ET_B agonists are inactive (Bax and Saxena, 1994).

The ET_B receptor was originally thought to mediate vasorelaxation, however the contraction of rabbit saphenous vein was shown to be mediated by ET_B receptors (Moreland *et al.,* 1992). From this, ET_B receptors have been subdivided into two types, ET_{B1}, located on the endothelial cells and mediating vasorelaxation through the release of nitric oxide (NO), and ET_{B2} receptors, located directly upon the vascular smooth muscle and mediating vasoconstriction (Sudjarwo *et al.,* 1993; Warner *et al.,* 1993a; Warner *et al.,* 1993b).

RECEPTOR COUPLING

Endothelin receptors belong to the superfamily of seven transmembrane domain-spanning receptors linked to G-proteins (for review see Davenport *et al.,* 1995). Endothelin receptors are coupled to several intracellular messengers which act to produce a wide variety of intracellular effects (see Figure 1.5). The major signal transduction pathway for the endothelin receptors has been established as activation of phosphatidylinositol 4,5-bisphosphate (PIP2)-specific phospholipase-C (PLC). Activation of PLC by endothelins leads to the cleavage of membrane bound phosphoinositides into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Each of these second messengers then subsequently activates separate signal transduction pathways. DAG activates protein kinase C (PKC), and IP_3 binds to a high affinity receptor on the endoplasmic reticulum and triggers the rapid mobilisation of Ca^{2+} (Sokolovsky, 1995). This increase in $[Ca^{2+}]_i$ can activate Ca^{2+} dependent protein kinases, in addition to Ca^{2+}-sensitive PLC and phospholipase-A_2 (PLA_2), which leads to the release of arachidonic acid (for reviews see Sokolovsky, 1992, Goto *et al.,* 1996).

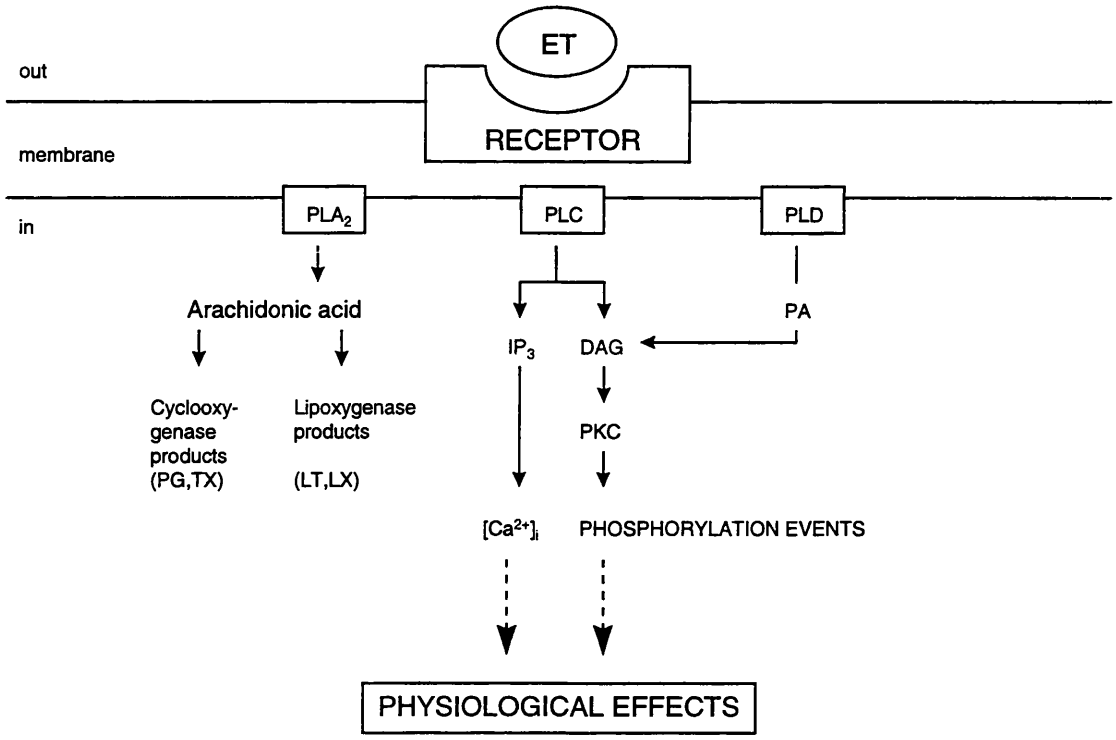


Figure 1.5. Schematic representation of some of the signal transduction pathways associated with activation of the endothelin receptor. DAG, diacylglycerol; IP_3, Inositol 1,4,5-triphosphate; LT, leukotriene; LX, lipoxin; PA, Phosphatidic acid; PG, prostaglandin; PKC, protein kinase C; PLA_2, phospholipase A_2, PLC, phosphoinositide specific phospholipase C; PLD, phospholipase D; TX, thromboxane.

ENDOTHELIN RECEPTOR ANTAGONISTS

Both selective and non-selective antagonists for ET_A and ET_B receptors have been developed (see Figure 1.6). The best known of the ET_A receptor antagonists is the cyclic pentapeptide BQ123 (Ihara *et al.,* 1992; see Figure 1.7). This compound was isolated from the fermentation broth of the bacteria *Streptomyces misakiensis.* A sulphonamide derivative BMS 182874 has been described (Webb *et al.,* 1995), as a potent orally active ET_A selective receptor antagonist.

Selective ET_B receptor antagonists have also been developed. These include BQ788 (Ishikawa *et al.,* 1994; see Figure 1.7), RES-701-1 (Karaki *et al.,* 1994) and IRL1038 (Urade *et al.,* 1992).

In addition, non-selective mixed ET_A/ET_B receptor antagonists have been described, including bosentan (Clozel *et al.,* 1994; see Figure 1.7) and SB209670 (Ohlstein *et al.,* 1994).

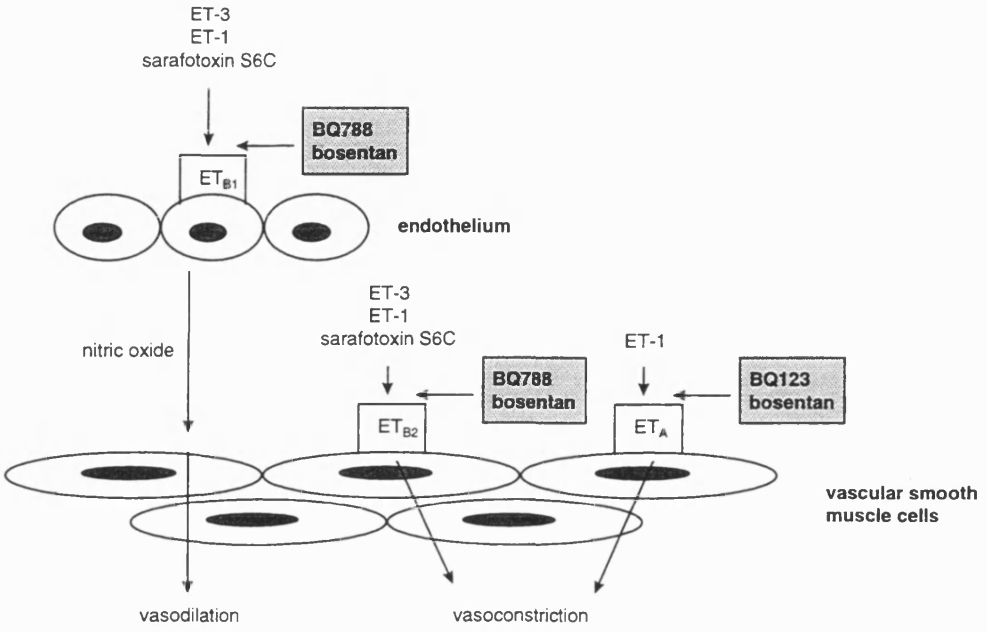
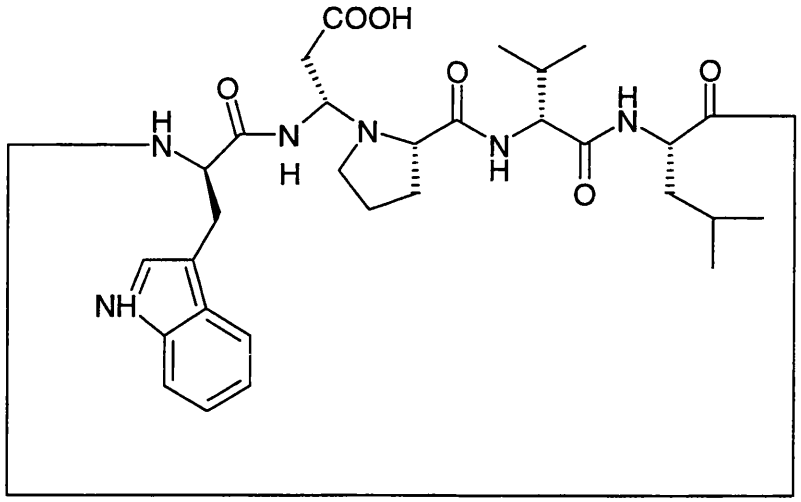
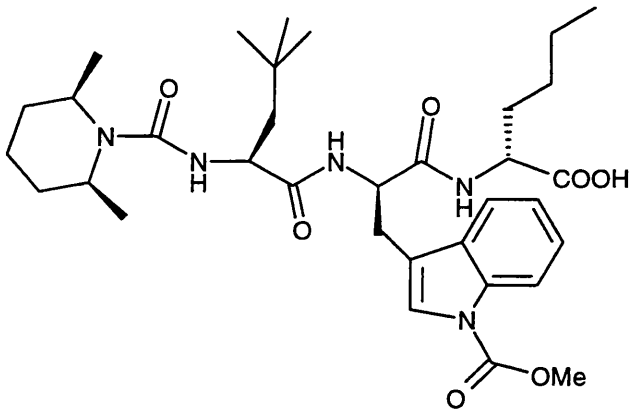


Figure 1.6. Location of the putative endothelin receptors and the agents which act upon them. Shaded blocks contain antagonists (modified from Douglas *et al.*, 1994).

a)



b)



c)

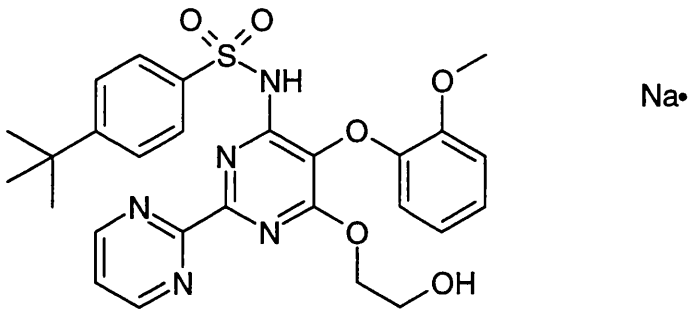


Figure 1.7. Structure of a) the endothelin ET_A receptor antagonist BQ123; b) the endothelin ET_B receptor antagonist BQ788; and c) the mixed ET_A/ET_B receptor antagonist bosentan (sodium salt).

PHARMACOLOGICAL ACTIONS OF ENDOTHELINS

Endothelins and sarafotoxins produce wide and diverse actions in both vascular and non-vascular smooth muscle (for reviews see Rubanyi and Polokoff, 1994; Levin, 1995). In the conscious rat, bolus administration of ET-1 causes a transient fall, followed by a prolonged increase in mean arterial blood pressure (Bird *et al.,* 1993; Filep *et al.,* 1993; McMurdo *et al.,* 1993). In isolated vessels, ET-1 causes contraction in both humans and animals, with a threshold of approximately 100 pM (Highsmith *et al.,* 1992) These contractions are mediated by both ET_A and ET_B receptors, and the responses can be blocked by the mixed ET_A/ET_B receptor antagonist bosentan (Seo *et al.,* 1994; White *et al.,* 1994).

Endothelins can also elicit relaxation of pre-constricted vessels *via* activation of ET_{B1} receptors, and the subsequent release of nitric-oxide (NO; Namiki *et al.,* 1992; Karaki *et al.,* 1993; Shetty *et al.,* 1993; Zellers *et al.,* 1994). Loesch and Burnstock (1995) have shown co-localisation of endothelin and nitric oxide synthetase (NOS) in endothelial cells from pulmonary and coronary arteries of new-born rats, and such ET/NO release may play an important role in the vasoregulation of the cardiac and pulmonary circulations. In addition to actions on the vasculature, endothelins are also potent constrictors of airway smooth muscle in both the bronchus and trachea (see Rubanyi and Polokoff, 1994). ET-1 has also been shown to promote

vascular and cardiac muscle proliferation and is a potent stimulator of DNA synthesis (see Barnes, 1994).

Endothelins are also oedemagenic in some vascular beds, including the bronchial circulation (Kurose *et al.,* 1993; Filep *et al.,* 1995, Lal *et al.,* 1995). However controversy still exists over the oedemagenic nature of endothelins in the pulmonary circulation, with some investigators finding ET-1 infusion augmenting albumin escape into the lung (Lehoux *et al.,* 1992; Zimmerman *et al.,* 1992) or raising the pulmonary vascular fluid filtration rate (Barnard *et al.,* 1991; Helset *et al.,* 1993). Other investigators have found no evidence that ET-1 by itself increases pulmonary vascular permeability to protein (Macquin-Mavier *et al.,* 1989; Rodman *et al.,* 1992; Sirosis *et al.,* 1992; Filep *et al.,* 1995) or pulmonary vascular fluid filtration coefficient (Barnard *et al.,* 1991; Horgan *et al.,* 1991). In addition to this, ET-1 does not augment albumin movement across cultured endothelial monolayers (Horgan *et al.,* 1991; Rodman *et al.,* 1992).

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF ENDOTHELINS

Endothelins have been postulated to be involved in the maintenance of basal vascular tone (Rubanyi, 1989); regulation of water balance (for review see Rubanyi and Shepherd, 1992); to have local and systemic roles in haemorrhage; and paracrine-, autocrine- and endocrine signalling (for review see Rubanyi and Polokoff, 1994).

Pathological conditions thought to involve endothelins include: coronary vasospasm (Matsuyama *et al.,* 1991; Toyo-oka *et al.,* 1991); cerebral vasospasm following sub-arachnoid haemorrhage (Mosaoka *et al.,* 1989; Lam *et al.,* 1991); Raynaud's disease (Zamora *et al.,* 1990); pre-eclampsia (Taylor *et al.,* 1990; Clark *et al.,* 1992); myocardial ischaemia (Yasuda *et al.,* 1990; Lam *et al.,* 1991); cerebral ischaemia (Ziv *et al.,* 1992); congestive heart failure (Lerman *et al.,* 1992; Stewart *et al.,* 1992; Tsutamoto *et al.,* 1994); shock syndrome (including septic shock, cardiogenic shock and haemorrhagic shock; see Rubanyi and Polokoff, 1994); bronchial asthma (Mattoli *et al.,* 1991); gastric ulceration (Peskar *et al.,* 1992); inflammatory bowel disease (Murch *et al.,* 1992); renal insufficiency and chronic renal failure (Koyama *et al.,* 1989; Warrens *et al.,* 1990; Deray *et al.,* 1992); hepatorenal syndrome (Uchihara *et al.,* 1992) and pulmonary hypertension and hypoxic vasoconstriction (Levy *et al.,* 1990; Stewart *et al.,* 1991).

The evidence for the involvement of ETs in pulmonary hypertension and pathological conditions that result in a reduction of pulmonary oxygen tension comes from several studies showing increased levels of ET-1 following hypoxia exposure, both in animals and humans.

Several animal models of pulmonary hypertension have been used to demonstrate elevated levels of ET-1. These models include idiopathic pulmonary hypertension in fawn hooded rats, where elevated plasma and lung levels of ET-1 were demonstrated, compared to normal control animals (Stelzner *et al.,* 1992), and monocrotaline-induced pulmonary hypertension in beagles, where plasma ET-1 levels increase by up to threefold in this form of chronic pulmonary hypertension (Okada *et al.,* 1995).

Further evidence for the involvement of ETs in pulmonary hypertension comes from studies utilising the ET receptor antagonists. Several studies have shown that the ET receptor antagonists BQ123 and bosentan can both prevent and halt the progression of the physiologic and morphologic changes that arise from exposure to chronic hypoxia (for review see Michael and Markewitz, 1996). In addition to this, it has been reported that BQ123 can prevent the rise in pulmonary artery pressure in rats exposed to hypoxia for a period of 90 min (Oparil *et al.,* 1995).

It has been shown that plasma levels of ET-1 are increased in patients with pulmonary hypertension associated with many different diseases, including primary pulmonary hypertension (Cacoub *et al.,* 1993); hypoxic lung disease (Stewart *et al.,* 1991); collagen vascular disease (Vancheeswaran *et al.,* 1994) and congestive heart failure (Cody *et al.,* 1992). One of the first studies showed an approximate six fold increase in plasma ET-1 levels in four patients with pulmonary hypertension (controls: 0.26 $\pm$ 0.24 pg/ml, n=14 vs 1.52 $\pm$ 0.45 pg/ml, n=4, $p<0.001$, Cernacek and Stewart, 1989). In addition to this, studies have shown a correlation between plasma ET-1 levels and the degree of increase in mean pulmonary artery pressure during hypoxia (Allen *et al.,* 1993). This study also demonstrated that there was no difference in the ET-1 levels in patients with chronic cardiopulmonary disorders without pulmonary hypertension.

PROJECT AIMS

(a) To develop a single pass hypoxic lung model, that allowed measurement of pulmonary perfusion pressure, pulmonary inflation pressure and lung weight.

(b) To investigate the role of endothelins in the responses to hypoxia in the lung.

(c) To develop a recirculating lung model that responded in the same way as a single pass model to hypoxia.

(d) To measure the release of ET-1 into the perfusate under both normoxic and hypoxic conditions.

(e) To measure prepro endothelin-1 mRNA levels within the lung following normoxic and hypoxic perfusion.

(f) To investigate the responses of the lung to exogenous ETs in the presence and absence of endothelin receptor antagonists.

SECTION 2

Materials and Methods

ANIMALS

Male Wistar rats (310 - 340g) were supplied by the University of Bath Animal House.

DRUGS AND CHEMICALS

Drugs and chemicals used were obtained from the following sources:

British Drug House (BDH), Poole:

Evans Blue dye

Life Technologies, Paisley:

Custom primers

Peninsula Laboratories, Merseyside:

Phosphoramidon

Peptide Institute, Osaka:

Big endothelin-1, Endothelin-1, Endothelin-3, Sarafotoxin 6C

Perkin Elmer (Applied Biosystems), Warrington:

10XPCR buffer II, Agarose, $MgCl_2$, *Taq* polymerase

Pharmacia Biotech, St Albans:

100 bp ladder, dATP, dCTP, dGTP, dTTP, dUTP, Oligo $d(T)_{12-18}$ primer, QuickPrep Micro mRNA purification kit

Promega, Southampton:

5x RT-buffer, M-MLV reverse transcriptase, RNAsin

R&D Systems, Abingdon:

Human ET-1 ELISA

Rhone Merieux LTD, Harlow:

Pentobarbitone sodium (Sagatal)

Sigma Chemical Company, Poole:

Acetylated bovine serum albumin, L-Arginine, Bovine Serum Albumin, Bradykinin acetate, Bromophenol blue, Colchicine, Cycloheximide, Ethidium bromide, Formamide, Gel loading buffer, Heparin Sulphate, Nω-nitro-L-Arginine, Nω-nitro-L-Arginine methyl ester, Phalloidin, Xylene cyanole FF

All other reagents were of analytical grade, and supplied by BDH, Poole; Fisons, Loughborough and Sigma Chemical Company, Poole.

The following compounds were kindly supplied by Drs A.G. Roach and T.J. Brown, Rhône-Poulec Rorer (Dagenham, England):

BQ123 (cyclo-[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]), BQ788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa) and bosentan (Ro470203; 4-tert-butyl-N-[6-(h-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulphonamide sodium salt)

PHYSIOLOGICAL SALT SOLUTION

A Krebs-Henseleit solution of the following composition (mM) was used: potassium chloride 4.7, potassium dihydrogen phosphate 1.2, calcium chloride 1.25, magnesium sulphate 1.2, sodium chloride 118, sodium bicarbonate 25, and glucose 11.1, pH 7.4. The Krebs solution was gassed with either 20%O_2/5%CO_2/75%N_2 (normoxic) or 95%N_2/5%CO_2 (hypoxic).

HEPARIN SOLUTION

Heparin sulphate (bovine) was dissolved in 0.9 % w/v saline to give 1000 IU ml^{-1}. Aliquots were stored at 4°C until use.

All other compounds were dissolved in sterile saline unless otherwise stated.

ISOLATED VENTILATED PERFUSED RAT LUNG

Lungs were isolated and perfused as described previously (Lal *et al.,* 1994). Male Wistar rats (310-340g) were anaesthetised with sodium pentabarbitone (100 mg/kg i.p.). Heparin (500 IU) was then administered i.v. *via* the tail vein; 5 min later the chest was opened and the pulmonary artery cannulated with a stainless steel cannula *via* the right ventricle. The left atrium and main mass of the ventricles were removed to allow free efflux of the perfusate. The trachea was cannulated and the lungs removed and placed in a warming jacket at 37°C. Lungs were perfused *via* the pulmonary artery at a constant rate of 5 ml min^{-1} with a Krebs-Henseleit solution, gassed with either 20%O_2/5%CO_2/75%N_2 (normoxic) or 95%N_2/5%CO_2 (hypoxic). All ventilation and perfusion tubing was of the low gas permeability type (Tygon R3603 and PharMed 65). Pulmonary perfusion pressure (PPP) was recorded *via* a pressure transducer (Druck model PDCR) connected to the pulmonary arterial cannula. The tracheal cannula was connected to a ventilator (Harvard miniature animal ventilator) and lungs were ventilated with room air at a stroke volume of 1ml and a rate of 28 inflations/min (with no positive end expiratory pressure). A pressure transducer (Druck model PDCR) attached to the tracheal cannula facilitated measurement of pulmonary inflation pressure (PIP). In addition lungs were suspended from a force displacement transducer (Dynamometer UF-1) for continual measurement of lung weight (see Figure 2.1). All responses were recorded on a Lectromed

MX6 pen recorder. Both normoxic and hypoxic control experiments were performed throughout the duration of the studies. Lungs were allowed to stabilise for 15 min prior to the start of the experiment.

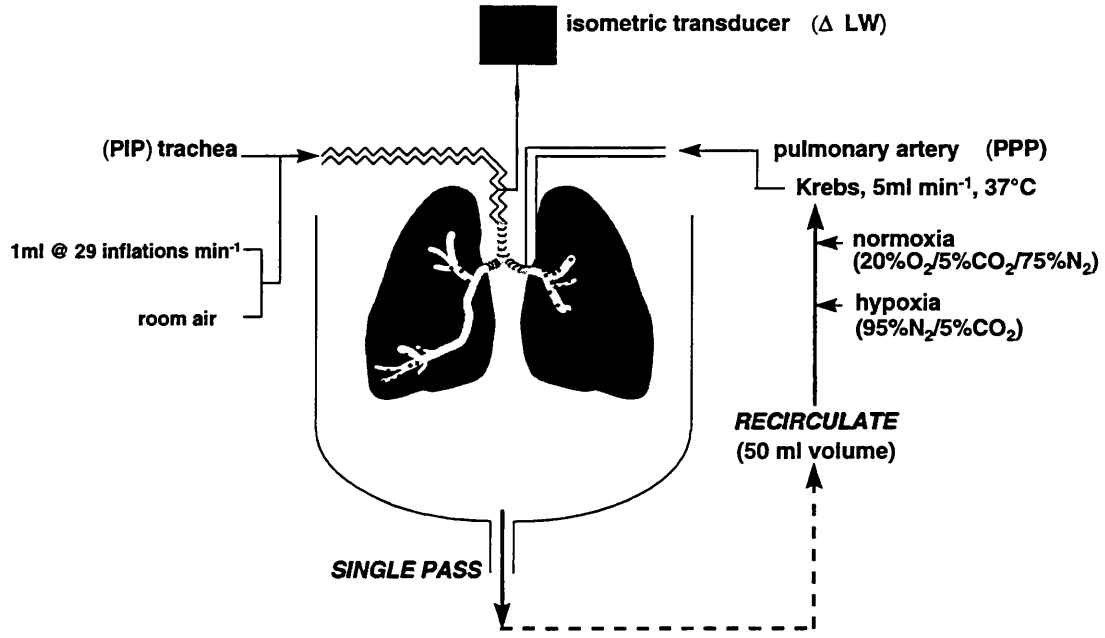


Figure 2.1. Schematic representation of the isolated, ventilated, perfused rat lung. LW, lung weight; PIP, pulmonary inflation pressure; PPP, pulmonary perfusion pressure.

AGONIST AND ANTAGONIST STUDIES

In a series of experiments the effects of a series of agonists was investigated. The lungs were allowed to stabilise for a period of 30 min. Then the agonist was administered *via* the pulmonary artery cannula in a bolus dose not exceeding 50 µl. The response was allowed to stabilise prior to administration of any subsequent dose.

In some experiments antagonists were added to the perfusate 15 min prior to agonist administration, and were present for the duration of the experiment.

HYPOXIA STUDIES

In a series of experiments the effect of systemic hypoxia on the lung was investigated. Two types of protocol were used, a single pass and a recirculating system.

Single-pass perfusion

Following the initial stabilisation period perfusion continued for a further 15 min prior to the onset of hypoxia. Hypoxia was initiated by switching to a Krebs-Henseleit solution previously equilibrated with 95%N_2/5%CO_2. In studies involving drug treatment, the drug was perfused for 15 min prior to the onset of hypoxia and for the remainder

of the hypoxic period (70 min). During the 15min pre-treatment period none of the agents used had any effect on basal PPP or LW. Control experiments (both normoxic and hypoxic) were performed throughout the duration of the study.

Recirculating perfusion

Following 15 min stabilisation, lungs were perfused in a recirculating manner (recirculating volume 50 ml) under normoxic conditions for a further 15 min. The lungs were then exposed to hypoxia by switching the gas mixture from 20%O_2/5%CO_2/75%N_2 to 5%CO_2/95%N_2. In studies involving drug treatment, the drug was added to the recirculating Krebs solution at the start of recirculation period. In keeping with the single pass perfusion study, control experiments (both normoxic and hypoxic) were performed throughout the duration of the study.

MEASUREMENT OF OXYGEN TENSION

In some experiments oxygen tension was measured in the perfusate entering and leaving the lung. The perfusate was pumped through a Clarke type oxygen electrode, calibrated to express PO_2 in mmHg. The electrode was calibrated with distilled water, which had been gassed with room air for 30 mins, to a PO_2 calculated from the following equation:

$$PO_2 \text{ (mmHg or Torr)} = \frac{20.93}{100} \times (b - vp)$$

Where b is the barometric pressure in mmHg, from a barometer, and vp is the water vapour pressure at any given temperature.

ALBUMIN BOUND DYE EXTRAVASATION

Evans Blue dye was added to a 10% w/v solution of bovine serum albumin to give a final concentration of 1 mg ml^{-1}. The mixture was dialysed overnight in 22/32 Visking tubing against an excess of distilled water. The albumin bound Evans Blue was then infused into the pulmonary artery at 50 µl min^{-1} for a 5 min period during the experiment. Following infusion, the lungs were perfused for a further 15 min to remove any remaining dye from the vasculature. Lungs were then dried overnight in an oven at 80°C. The trachea was then removed, and the weight of the main mass of the lungs recorded. Lungs were then digested in 5 ml formamide at 37°C for 20 hours. The formamide was then removed, and centrifuged (6000 xg, room temperature, 30 min; IEC Centra-M). The absorbance of standard concentrations of Evans Blue (0.001 - 100 µg ml^{-1}) or lung extracts were measured at 622 nm in a spectrophotometer (Pye Unicam, PU 8610), against an appropriate formamide blank. The concentration of the dye in the lung extracts was calculated by reference to a standard curve of Evans Blue concentrations. From this the amount of Evans Blue retained in the lung could be calculated.

ENDOTHELIN EXTRACTION

Endothelins were extracted from both perfusate samples and tissue samples. Two extraction procedures were used:

Perfusate extraction

ETs were extracted from the recirculated perfusate using a modified acetic acid extraction (Rolinski *et al.,* 1994). 25 ml of perfusate was acidified with glacial acetic acid to give a final concentration of 10% v/v, and the sample centrifuged (2500 xg, room temperature, 15 min).

Tissue extraction

ETs were extracted from tissue samples using a modified acetic acid extraction (Clerico *et al.,* 1994). Approximately 500 mg wet weight of tissue was homogenised in 5 ml 10% acetic acid (Ultra-Turrax, 20000 rpm, 30 sec). The sample was then boiled for 10 min and allowed to cool to room temperature, and centrifuged to produce a clear supernatant (25000 xg, 30 min, 4°C).

The ET extraction from the perfusate and tissue samples then followed the same procedure. The supernatant was applied to a washed (3 ml methanol, 3 ml distilled water, 3 ml 10% v/v acetic acid) Amprep ethyl C2 minicolumn under negative pressure. The column was then washed with 3ml 10% v/v acetic acid and 6ml ethyl acetate. The ETs

were eluted from the column with 3ml methanol : 0.05 M ammonium bicarbonate 80/20 v/v, and dried overnight in a vacuum oven.

ET-1 ASSAY

ET-1 was measured in an R&D Systems Human ET-1 ELISA. Dried samples were reconstituted in 250 µl sample buffer and ET-1 was quantitated with a R&D Systems Human ET-1 ELISA. Reconstituted samples were assayed within one hour, and assayed in duplicate and results measured on a plate reader (Titertek Multiskan MC340). The ELISA is sensitive to <1.0pg ml^{-1} ET-1 and shows <1% cross reactivity with big ET-1 and 14% cross reactivity with ET-3.

PROTEIN DETERMINATION

Protein levels were determined from the homogenised tissue samples using the Bradford method. Bovine serum albumin (1-10 mg/ml) was used to produce a standard curve.

mRNA EXTRACTION

Total lung mRNA was extracted using a Pharmacia Biotech QuickPrep Micro mRNA purification kit.

REVERSE TRANSCRIPTION AND 1st STRAND cDNA SYNTHESIS

Reverse transcription (RT) of the isolated mRNA was performed to obtain cDNA for polymerase chain reaction (PCR) and analysis. Briefly, 2µl oligo $d(T)_{12-18}$ primer (50 µM) was added to 20 ng poly(A+) mRNA in 10 µl sterile distilled water (SDW) and heated to 65°C for 10 min, then allowed to cool to room temperature for 2 min and placed on ice. 8 µl of pre-prepared reaction mixture containing 4µl 5x RT-buffer (250 mM Tris-HCl pH8.3, 375 mM KCl, 15mM $MgCl_2$, 50 mMDTT), 1µl acetylated bovine serum albumin (2 mg ml^{-1}), 1µl dNTPs (20mM, 1 part each 100mM dATP, dCTP, dGTP, dUTP and 1 part sterile distilled water), 1µl RNAsin (20-40 U μl^{-1}), 1µl M-MLV reverse transcriptase (200 U μl^{-1}) was then added, and the reaction mixture was mixed by inversion and incubated at 37°C for 60 min. 40µl of sterile distilled water was added at the end of the incubation and the cDNA stored at -20°C.

PCR

The primer sets for rat prepro ET-1 mRNA were designed on PRIMER SELECT and LASER GENE (DNA Star, Wisconsin, USA). For the amplification of cDNAs the following primers were used. β-actin: primer I, 5'-ccT Agc Acc AAg AAg ATc AA-3', primer II, 5'-TTT cTg cgc AAg TTA ggT TTT gTc AA-3', annealing temperature (AT) 50°C; rat prepro-endothelin-1: primer I, 5'-gAg gcc ATc Agc AAc Agc ATc A-3', primer II, 5'-Tcc gAg gcc ATc ccc AgA c-3', AT 58°C. Reactions were performed in 100 µl volumes containing 1xPCR buffer II, 1.5mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 0.4 µM primer I, 0.4 µM primer II, 2.5 U *Taq* polymerase and 5 µl of the RT reaction. The reaction mixtures were incubated at 94°C for 5 min and then run for 31 cycles for the β-actin, and 35 cycles for the rat prepro-endothelin-1, at 94°C for 1min, AT for 1 min and 72°C for 2 min, finishing with a 6 min incubation at 72°C in a Perkin Elmer DNA 480 thermal cycler. Sterile distilled water controls were included with each batch of reactions.

Twenty microlitres of each PCR mixture was mixed with 2 µl gel loading buffer (0.042% w/v bromophenol blue; 0.042% w/v xylene cyanole FF; 6.7% w/v sucrose), and 14 µl electrophoresed on a 2% agarose gel. Gels were stained with ethidium bromide and photographed.

STATISTICAL ANALYSES

Increases in PPP and LW induced by hypoxia are expressed as actual recorded values recorded at set time points following the onset of hypoxia. Increases in PPP, PIP and LW induced by bolus injections of agonists are expressed relative to the basal values prior to the addition of each dose. Data were expressed as mean ± standard error of the mean (SEM). Data from control experiments was pooled for each series of experiments, and the pooled data used in the statistical analysis. Analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* test or Student's (unpaired) t-test were used to test the level of significance as appropriate. Probability values of $p < 0.05$ were considered significant.

SECTION 3

Results

MODEL VALIDATION

The use of the salt perfused isolated ventilated rat lung has been characterised previously (Lal, 1995). However the use of an isolated lung with a perfusate (systemic) hypoxia has not previously been examined.

PO_2 DETERMINATION

The use of different types of tubing on the experimental apparatus led to the observation of changes in perfusate PO_2, dependent upon not only the gas mixture used to gas the Krebs buffer, but also upon the tubing used. Standard PVC laboratory tubing (Portex) allowed diffusion of oxygen into the perfusate, thus altering the PO_2 level between the Krebs reservoir and the lungs. To alleviate this problem low oxygen permeability tubing (Tygon R3060 and PharMed 65) was used, which minimised oxygen diffusion.

In addition to this, several perfusion/ventilation regimens were tried, the results of which can be seen in table 3.1. The arterial PO_2 value equates to the PO_2 of the Krebs in the reservoir prior to perfusion and the venous PO_2 was measured in perfusate following efflux from the pulmonary vein.

Table 3.1. Perfusate PO_2 values under several gassing regimens.

Krebs gassed with:	Lungs ventilated with:	Arterial PO_2 (mmHg)	Venous PO_2 (mmHg)
O_2/CO_2 (95%/5%)	Room Air	580	165
O_2/CO_2 (95%/5%)	N_2/CO_2	Not Measured	60
N_2/CO_2 (95%/5%)	Room Air	15	100
N_2/CO_2 (95%/5%)	N_2/CO_2	Not Measured	5
$N_2/O_2/CO_2$ (75%/20%/5%)	Room Air	140	135
$N_2/O_2/CO_2$ (75%/20%/5%)	N_2/CO_2	Not Measured	40

The values obtained in the above table were determined during an initial series of experiments to determine the perfusion/ventilation regimen to be used. The values quoted in the text are taken from subsequent measurements made during the course of the study. The eventual regimen used involved gassing the Krebs with a 75%/20%/5% mixture of $N_2/O_2/CO_2$ for normoxic perfusion, as this provides a more physiological oxygen tension than a 95%/5% mixture of O_2/CO_2. A 95%/5% mixture of N_2/CO_2 was utilised for the hypoxic perfusion. The lungs were ventilated with room air for all experiments. This resulted in PO_2 levels (determined at the pulmonary arterial cannula) of 5.5 ± 1.9 mmHg under a hypoxic conditions (95% N_2 / 5% CO_2) and 121 ± 7.3 mmHg under normoxic conditions (20% O_2 / 5% CO_2 / 75% N_2; n=3-6).

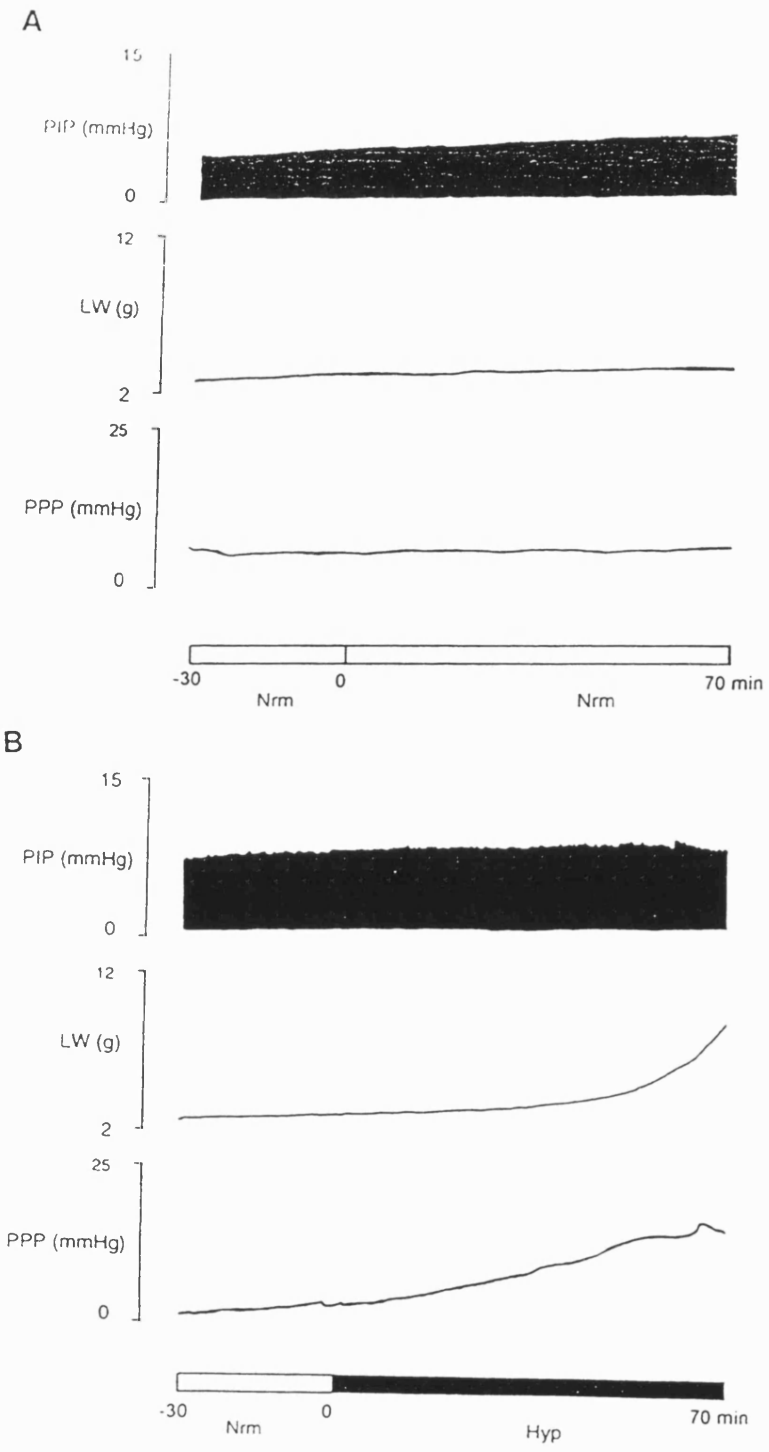
SINGLE PASS PERFUSION

Basal Parameters

Following the 15 min stabilisation period, under normoxic conditions, PPP was 6.54 ± 0.16 mmHg and the increase in LW, over the 15 min stabilisation period, was 0.06 ± 0.01 g (n=72).

Effects of Hypoxia

In lungs perfused with normoxic Krebs solution, 70 min perfusion resulted in an increase in PPP from 6.6 ± 0.6 mmHg to 8.4± 0.5 mmHg, and an increase in LW of 0.4 ± 0.1 g (n=7). Neither of these changes were significant. However, in lungs which were perfused for 70min with hypoxic Krebs solution, PPP increased from 8.2 ± 0.8 mmHg to 15.4 ± 1.4 mmHg (n=10, $p<0.001$) and LW increased by 2.4 ± 0.6 g (n=10, $p<0.001$; see Figure 3.1 and Figure 3.2). The increase in PPP was slow and gradual. LW began to increase following approximately 30 min hypoxic perfusion, with an initial gradual increase followed by a rapid increase. Both of these changes are also significantly greater than those seen over the same time period in the control normoxic group ($p<0.001$). Over the time course of these experiments there was no significant difference in PIP in either normoxic or hypoxic lungs, therefore the data has been omitted.


A
15
PIP (mmHg)
0
12
LW (g)
2
25
PPP (mmHg)
0
-30
0
70 min
Nrm
Nrm
B
15
PIP (mmHg)
0
12
LW (g)
2
25
PPP (mmHg)
0
-30
0
70 min
Nrm
Hyp

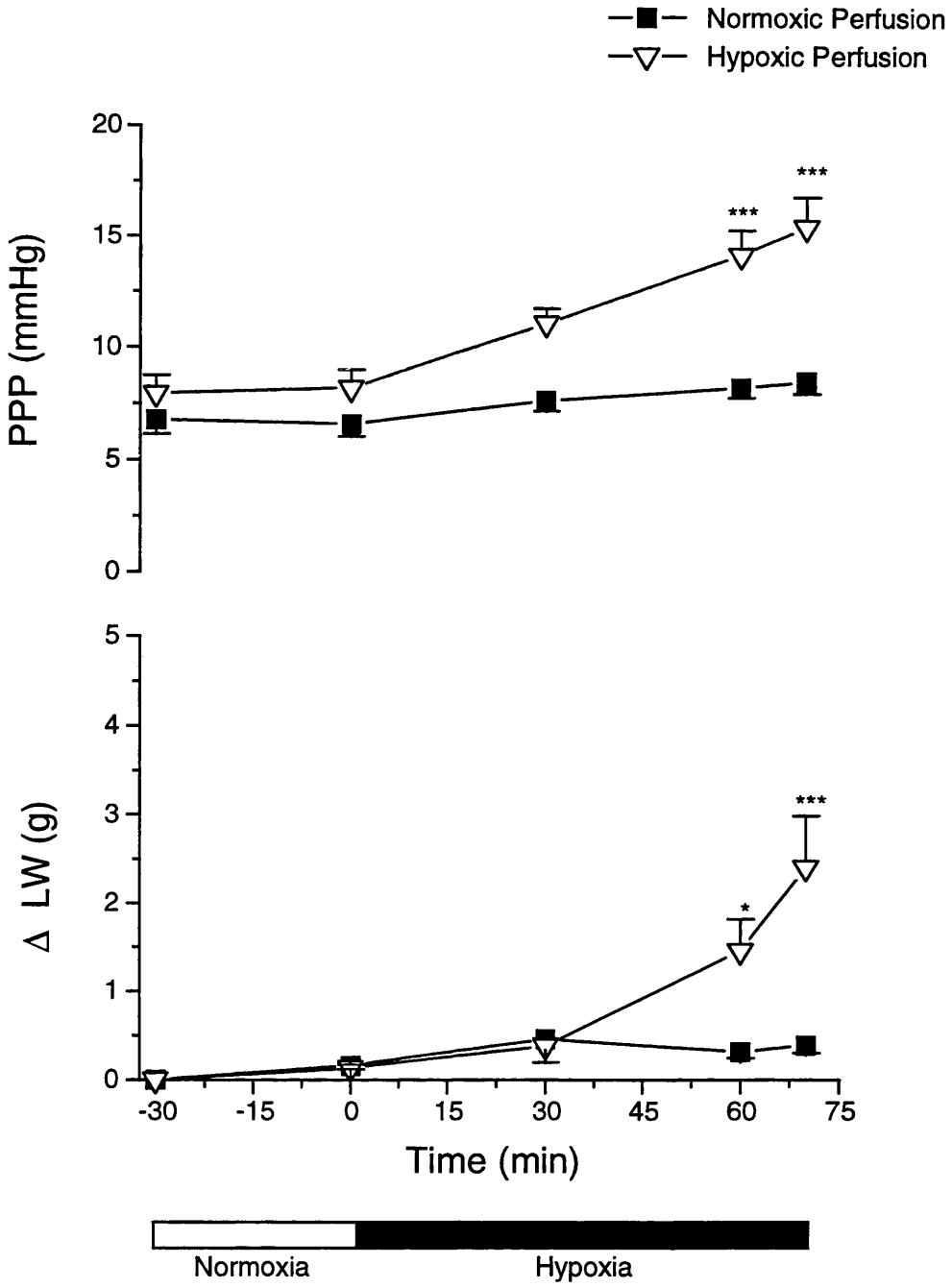


Figure 3.2. The effects of Normoxic and Hypoxic perfusion on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in the isolated ventilated perfused rat lung. n=7-10, * $p<0.05$; *** $p<0.001$ vs time-matched normoxic controls.

EFFECTS OF INHIBITORY AGENTS ON HPV

(a) Prazosin

Prazosin (100 nM), at a concentration that was shown to block responses to 25 nmol phenylepherine, did not prevent the development of HPV in the lung. PPP increased by 5.5 mmHg (n=2) in prazosin treated lungs, compared to a 7.2 ± 1.4 mmHg (n=10) increase in the hypoxic control lungs. In addition to this the increase in lung weight observed in the prazosin treated animals was 4.6g, compared to an increase of 2.4 ± 0.6 g (n=10) in the hypoxic control over the same time period (results not shown).

(b) Indomethacin

Addition of indomethacin (3μM) to the perfusate 15 min prior to the onset of hypoxia resulted in the attenuation of hypoxia-induced increase in perfusion pressure. This was accompanied by a reduction in the hypoxia induced increase in LW, although this was not significant (see Figure 3.3).

(c) Nitro-L-arginine, L-NAME and L-arginine

The nitric oxide synthetase inhibitors nitro-L-arginine (100μM) and L-NAME (100μM) had no effect upon basal parameters following 15 min perfusion, and had no significant effect upon the hypoxia-induced increase in vascular resistance, however, L-NAME (100μM) significantly attenuated the increase in lung weight caused by hypoxia (from 2.4 ± 0.6 g to 0.51 ± 0.14 g, n=5-10, $p<0.001$; see Figure 3.4).

Addition of 500μM L-arginine to the perfusate, in the absence of nitro-L-arginine and L-NAME, resulted in an attenuation of the increase in vascular resistance following hypoxia from 15.4 ± 1.4 mmHg to 9.6 ± 1.7 mmHg (n=4-10, $p<0.05$; see Figure 3.4).

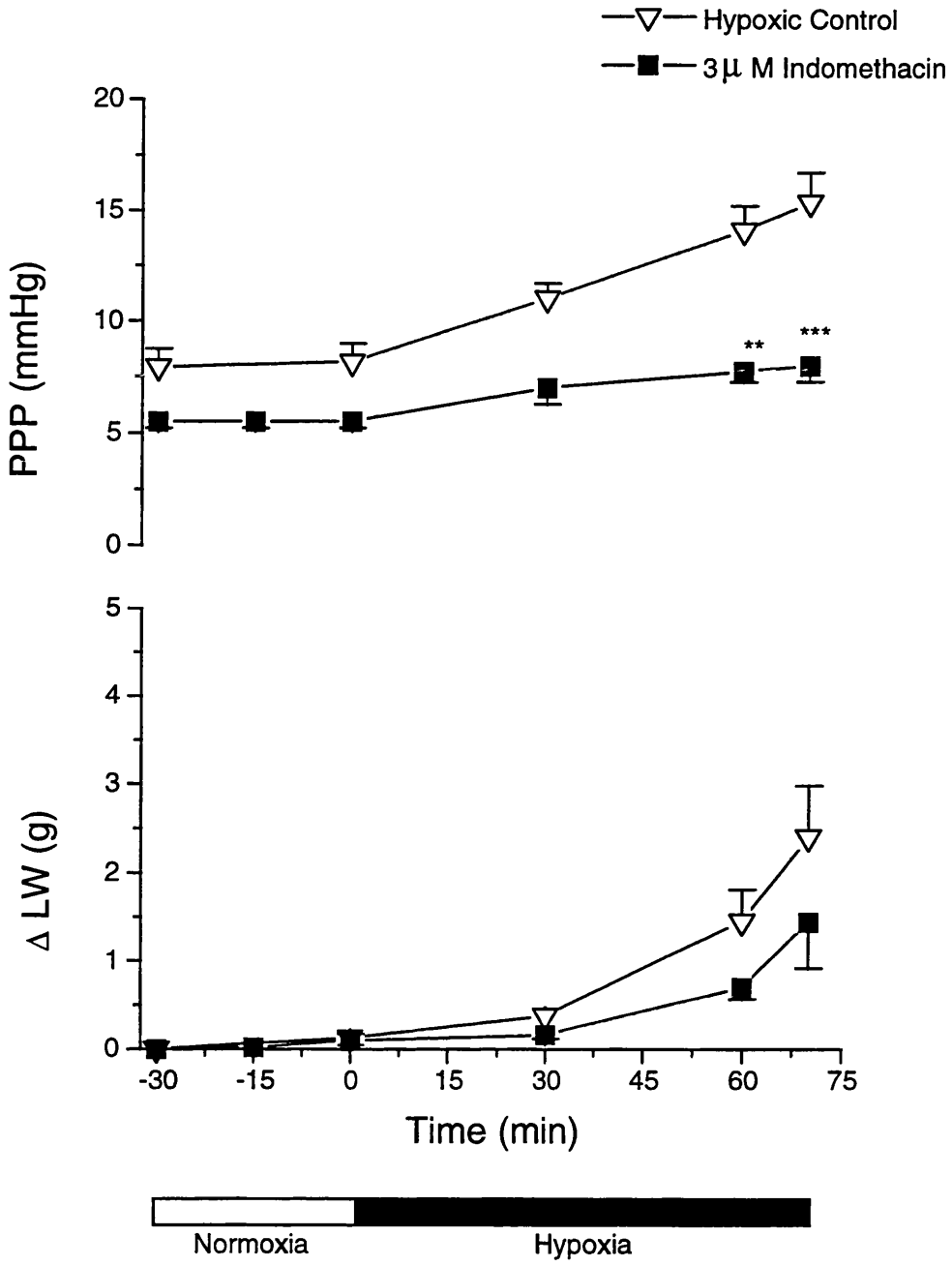


Figure 3.3. The effect of 3μM Indomethacin upon Hypoxia induced increases in Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in the isolated ventilated perfused rat lung. n=4-10, ** $p<0.01$, *** $p<0.001$ vs time-matched hypoxic control.

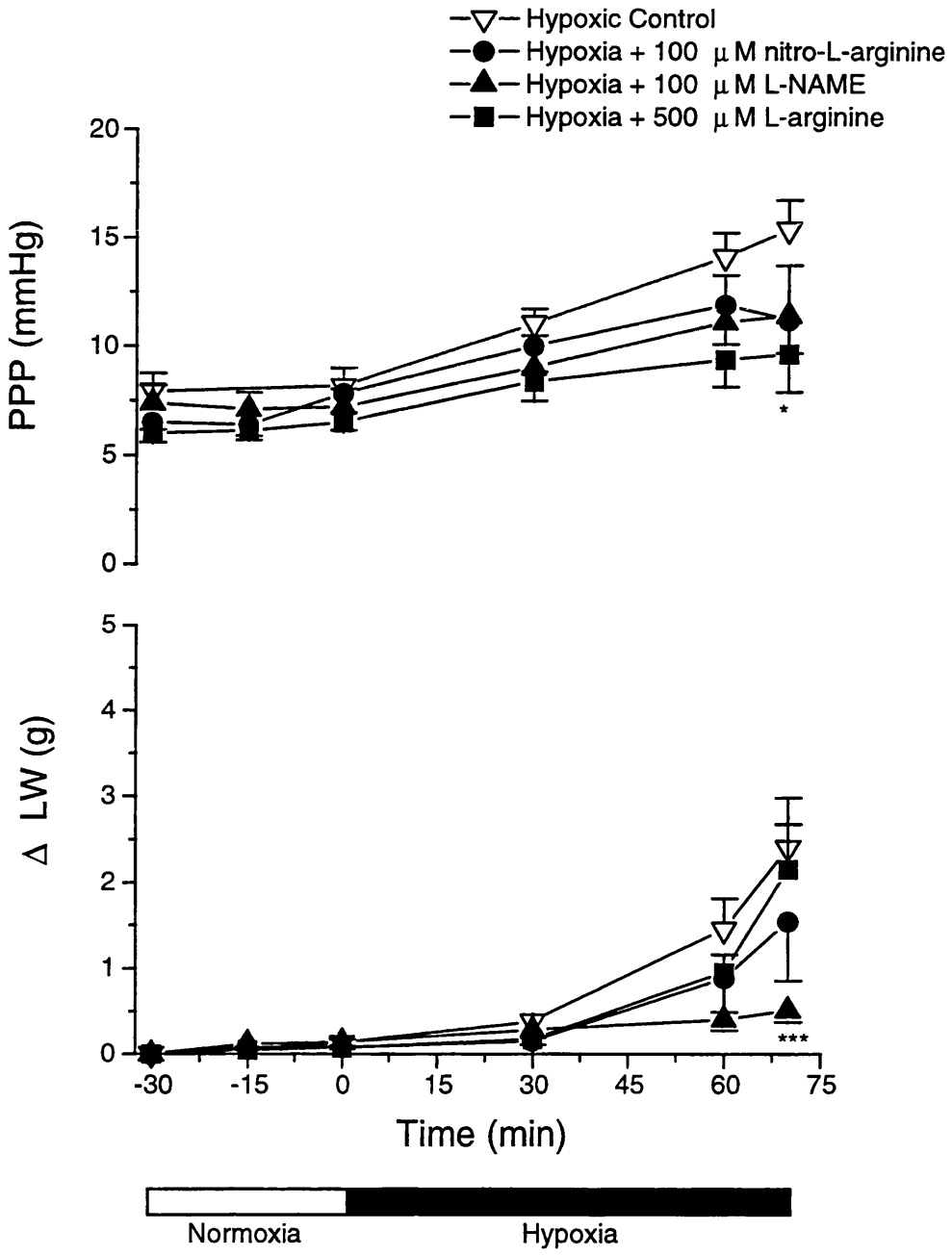


Figure 3.4. The effect of Nitro-L-arginine, L-NAME and L-arginine upon Hypoxia induced increases in Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in the isolated ventilated perfused rat lung. n=4-10, * $p<0.05$; *** $p<0.001$ vs time-matched hypoxic controls.

EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISTS ON RESPONSES TO HYPOXIA

To examine the involvement of the endothelins in the increase in vascular resistance and increase in lung weight following the onset of hypoxia, a variety of drugs which inhibit ET formation, secretion and actions were studied.

(a) BQ123

BQ123, an ET_A receptor antagonist, at concentrations of 3μM and 10μM, reduced the increases in PPP evoked by hypoxia from 15.4 ± 1.4 mmHg to 7.8 ± 0.4 and 9.2 ± 0.8 mmHg, respectively (n=6-10, $p<0.001$). BQ123 also attenuated the hypoxia-induced increase in LW from 2.4 ± 0.6 g to 2.1 ± 0.4 and 1.0 ± 0.2 g in a concentration-dependent manner (n=6-10), however, when compared to time-matched hypoxic controls, this reduction in LW was only significant at the higher concentration used ($p<0.01$; see Figure 3.5).

(b) BQ788

BQ788, an ET_B receptor antagonist, at a concentration of 3μM, reduced the hypoxia-induced rise in PPP (from 15.4 ± 1.4 mmHg to 9.6 ± 0.4 mmHg; n=5-10, $p<0.01$) when compared to time-matched hypoxic controls (see Figure 3.6). In addition BQ788 attenuated the hypoxia-induced increase in LW from 2.4 ± 0.6 g to 0.6 ± 0.1g (n=5-10, $p<0.001$).

(c) Bosentan

The mixed ET_A/ET_B receptor antagonist bosentan (1.5 and 5 μM) also significantly attenuated the hypoxia-induced increases in LW from 2.4 ± 0.6 g to 0.85 ± 0.1 (1.5 μM) and 0.5 ± 0.1 g (5 μM; n=4-10, $p<0.01$) when compared to time-matched hypoxic controls. However, only the lower concentration of bosentan (1.5μM) produced a significant inhibition of the HPV (from 15.4 ± 1.4 mmHg to 9.0 ± 1.2 mmHg, n=4-10, $p<0.05$) when compared to time-matched hypoxic controls (Figure 3.7).

EFFECTS OF AN ENDOTHELIN CONVERTING ENZYME INHIBITOR ON RESPONSES TO HYPOXIA

Phosphoramidon

As can be seen from Figure 3.8, the ECE inhibitor, phosphoramidon (1μM), significantly reduced both the hypoxia-induced vasoconstriction, from 15.4 ± 1.4 mmHg to 8.9 ± 0.8 mmHg (n=4-10, $p<0.01$), and increase in LW, from 2.4 ± 0.6 g to 1.1±0.3 g (n=4-10, $p<0.05$), when compared to time-matched hypoxic controls.

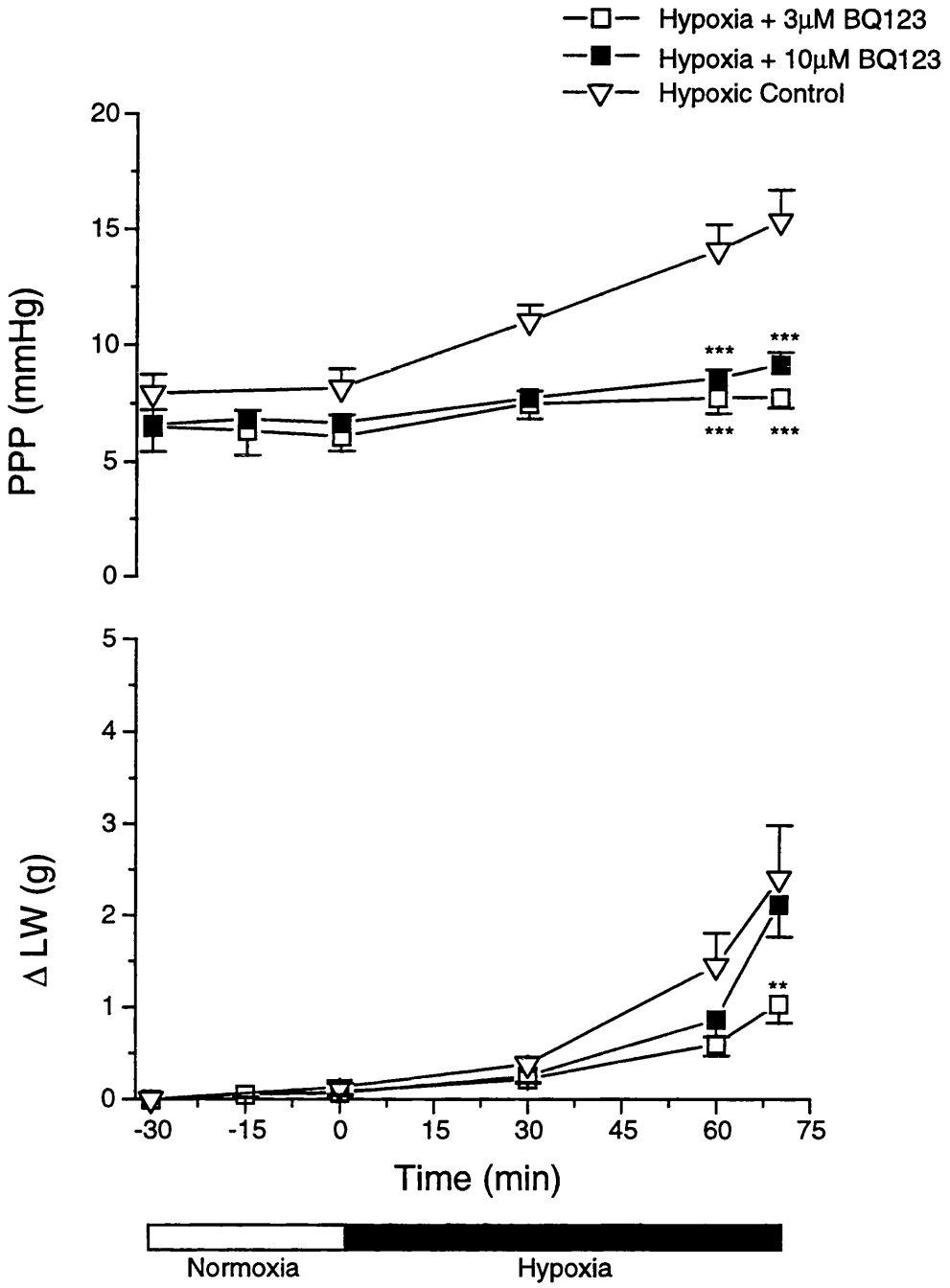


Figure 3.5. The effect of BQ123 (3 and 10 μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=6-10, ** $p<0.01$; *** $p<0.001$ vs hypoxic control.

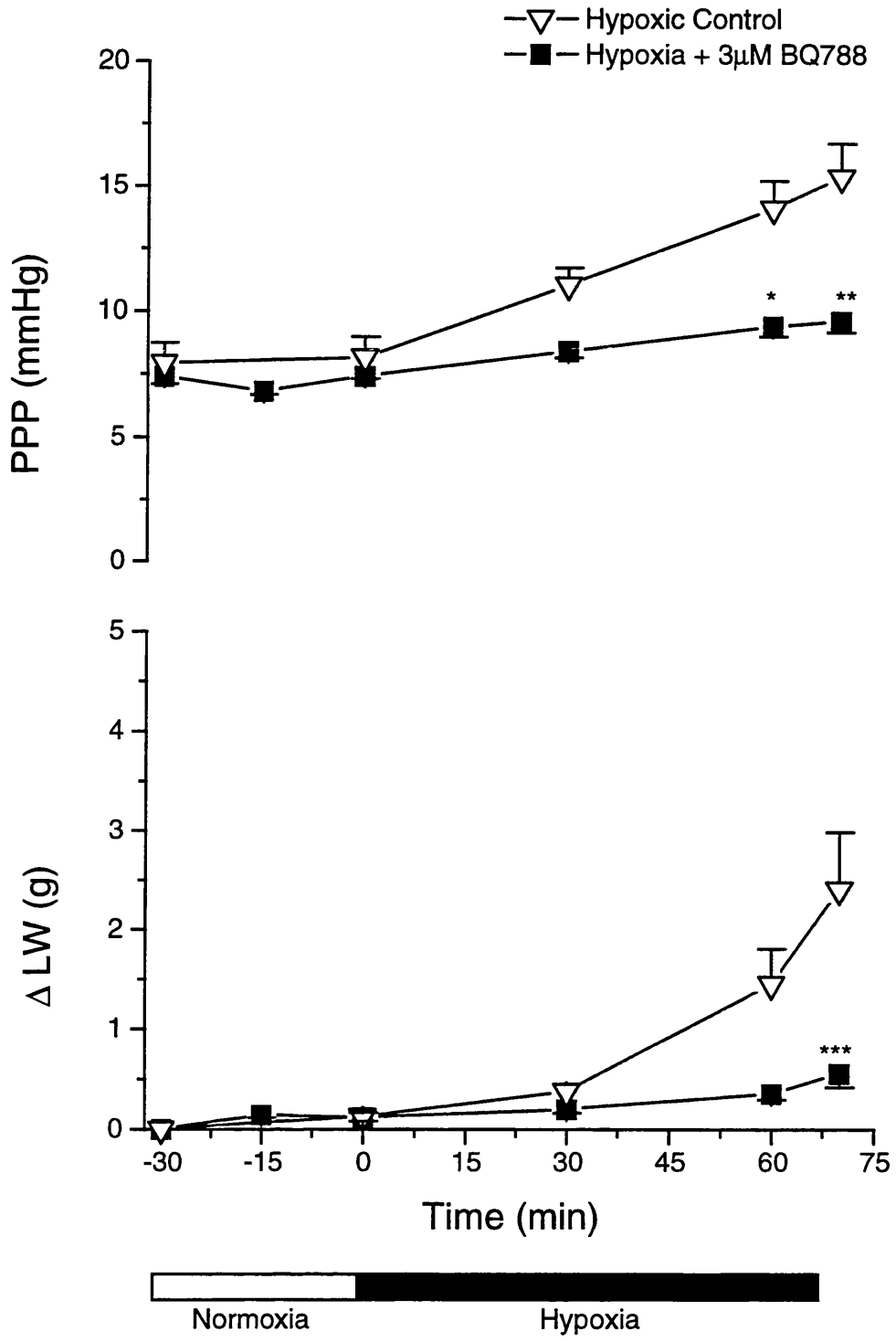


Figure 3.6. The effect of BQ788 (3μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=5-10, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs hypoxic control.

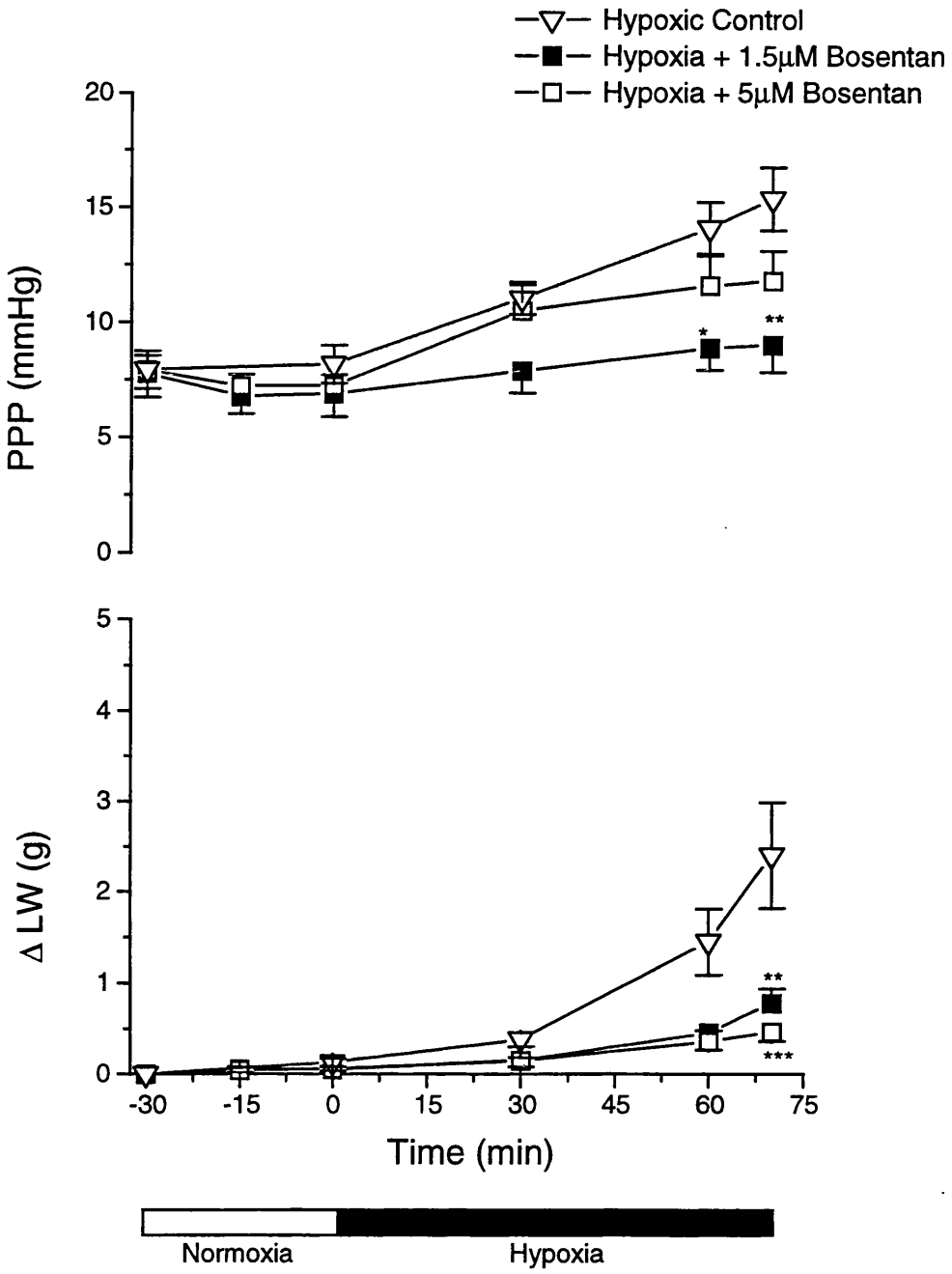


Figure 3.7. The effect of Bosentan (1.5 and 5 μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=4-10, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs hypoxic control.

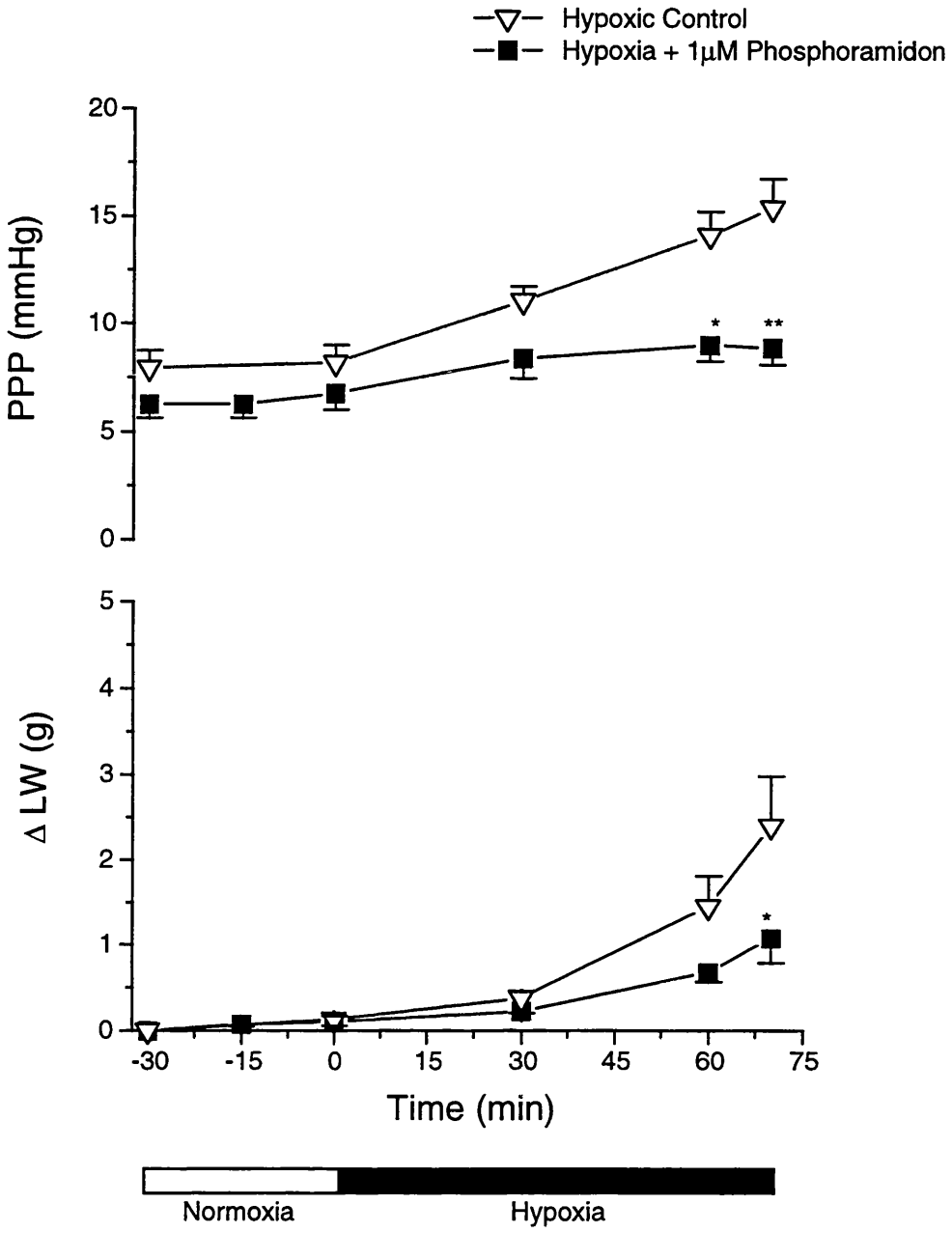


Figure 3.8. The effect of Phosphoramidon (1μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=4-10, * $p<0.05$; ** $p<0.01$ vs hypoxic control.

EFFECTS OF CYTOSKELETAL MODIFYING AGENTS ON RESPONSES TO HYPOXIA

Phalloidin and colchicine

When compared to time-matched hypoxic controls, phalloidin (10 and 50 nM), a f-actin stabiliser, and the microtubule-disrupting agent colchicine (100nM) attenuated the hypoxia-induced increases in PPP from 15.4 ± 1.4 mmHg to 8.8 ± 1.1, 9.9 ± 0.8 and 8.4 ± 0.6 mmHg, respectively, n=5-10, $p<0.01$, (see Figure 3.9). Colchicine, and the higher concentration of phalloidin, also significantly lowered the increases in LW from 2.4 ± 0.6 g to 0.7 ± 0.2 and 0.2 ± 0.1 g respectively (n=5-10, $p<0.001$, see Figure 3.10). In some experiments bradykinin (40 nmol, n=2 for both phalloidin and colchicine) was added prior to the addition of phalloidin and colchicine and at the end of the experiment to assess possible changes in vascular reactivity caused by these agents. In these experiments the increase in PPP elicited by bradykinin was the same at the start and end of the experiment (10.8 vs 12.5 mmHg for phalloidin and 9.5 vs 14.0 mmHg for colchicine).

EFFECTS OF A PEPTIDE SYNTHESIS INHIBITOR ON THE RESPONSES TO HYPOXIA

Cycloheximide

The protein synthesis inhibitor cycloheximide (5 μM) significantly lowered increases in PPP evoked by hypoxia, from 15.4 ± 1.4 mmHg to 6.9 ± 0.4 mmHg, and LW, from 2.4 ± 0.6 g to 0.65 ± 0.2 g (n=4-10, $p<0.01$), when compared to time-matched hypoxic controls (see Figure 3.11).

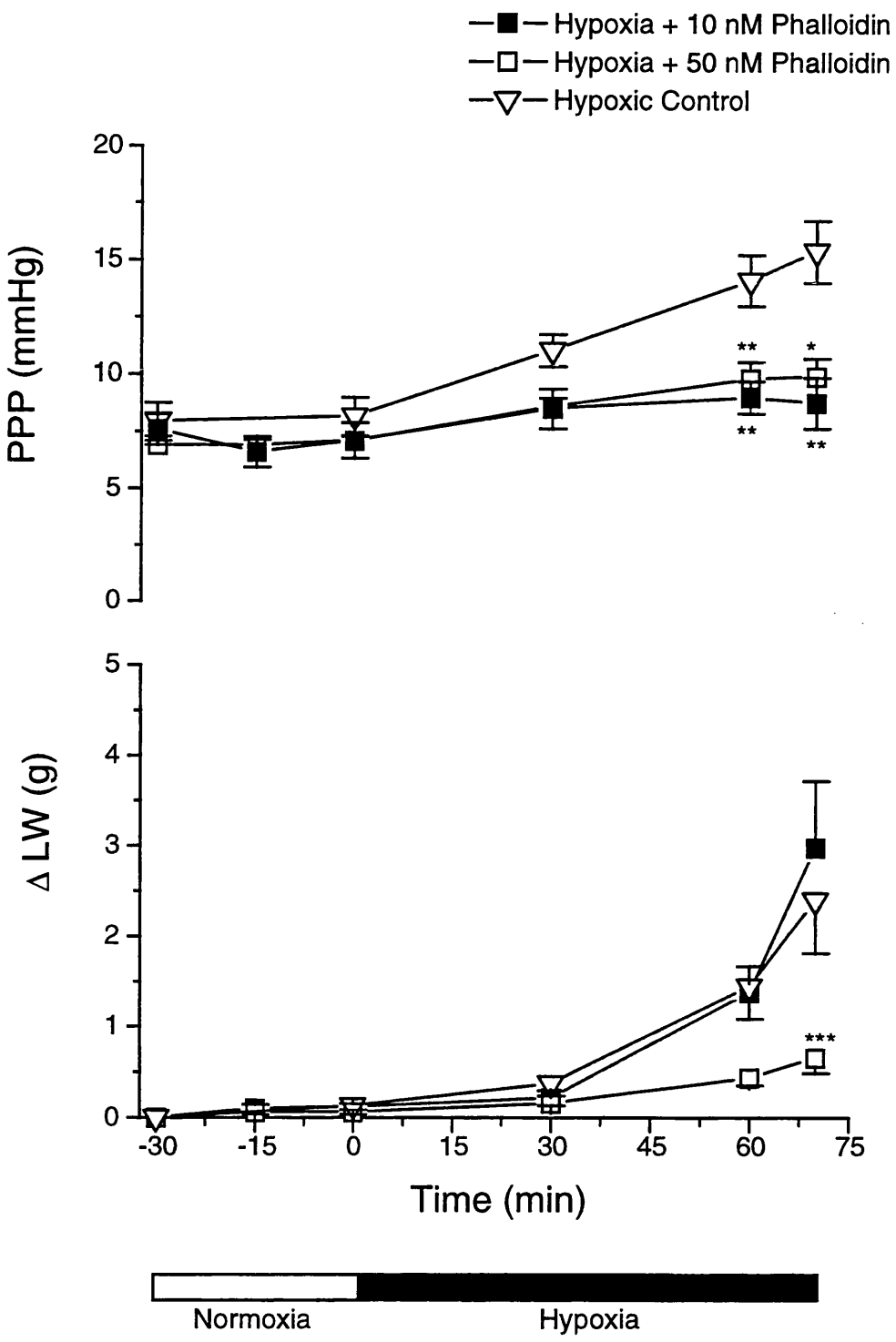


Figure 3.9. The effect of Phalloidin (10 and 50 nM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=5-10, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs hypoxic control.

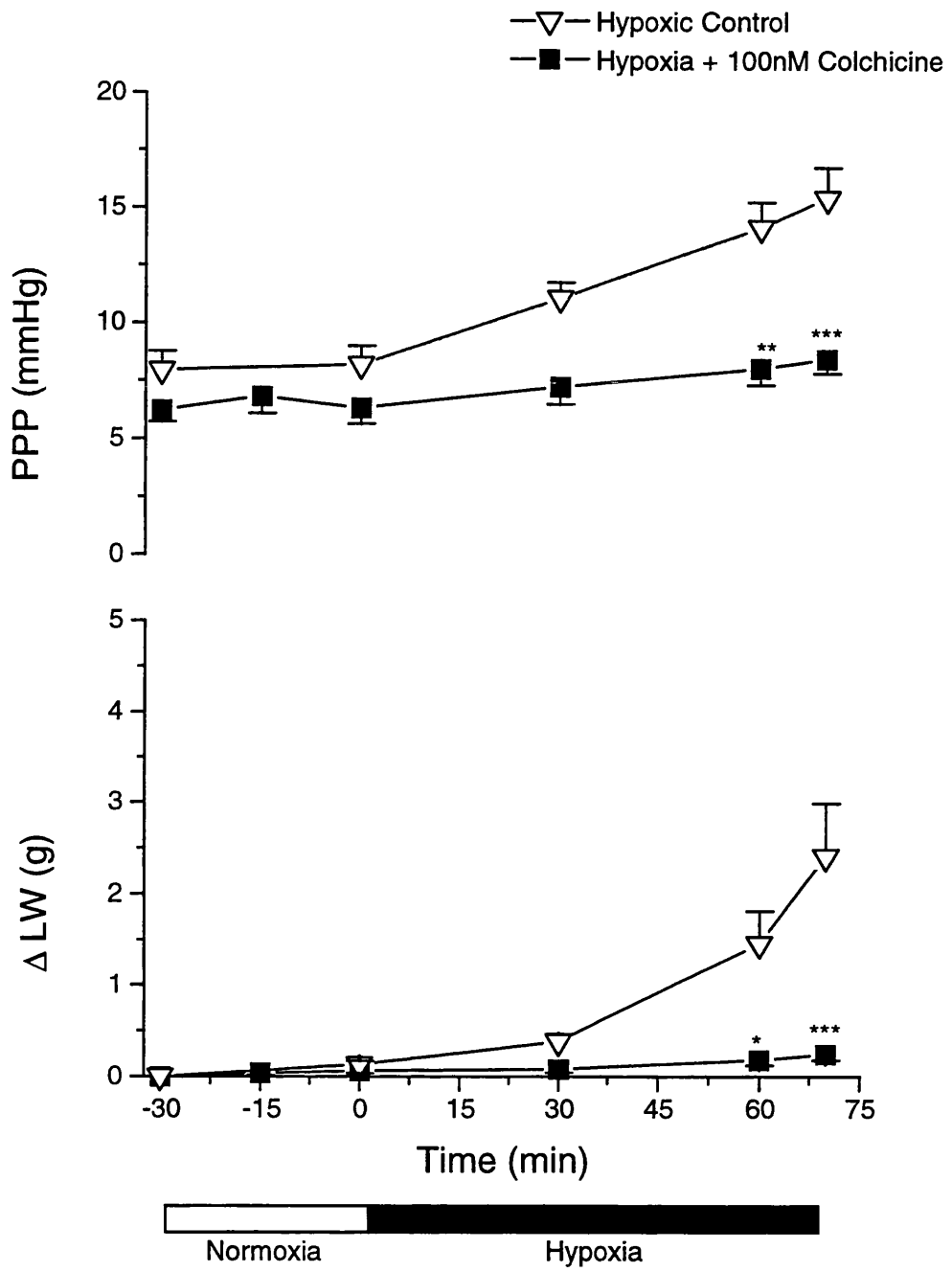


Figure 3.10. The effect of Colchicine (100 nM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=5-10, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs hypoxic control.

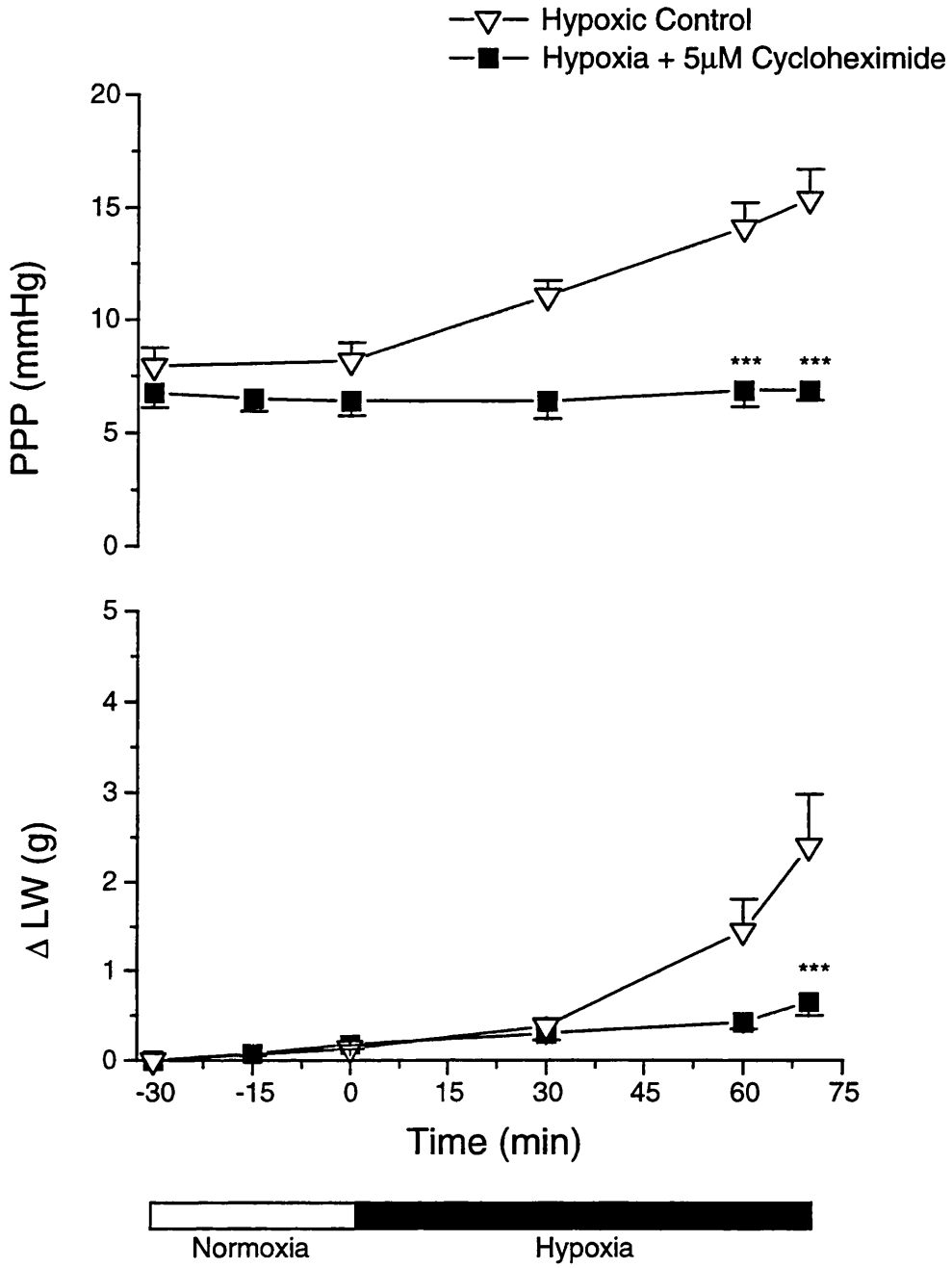


Figure 3.11. The effect of Cycloheximide (5μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated perfused rat lung. n=4-10, *** $p<0.001$ vs hypoxic control.

EVANS BLUE DYE ACCUMULATION

To investigate the possible mechanisms involved in the change in LW seen following hypoxic perfusion, a series of experiments were performed utilising albumin bound Evans blue dye. With hypoxic perfusion, the increase in LW was associated with a large accumulation of Evans blue (29.6 ± 6.1 ng mg^{-1} dry weight, n=6) when compared to time-matched normoxic controls (0.98 ± 0.09 ng mg^{-1} dry weight, n=5, $p<0.01$). Back-calculation of the fluid retention from the retained dye indicated that the lungs retained 1.7 ± 0.4 ml of fluid during the dye infusion (n=6). Assuming a specific gravity of 1.0, this does not differ significantly from the recorded increase in LW of 1.8 ± 0.6 g (n=6) during the infusion period (see Figure 3.12).

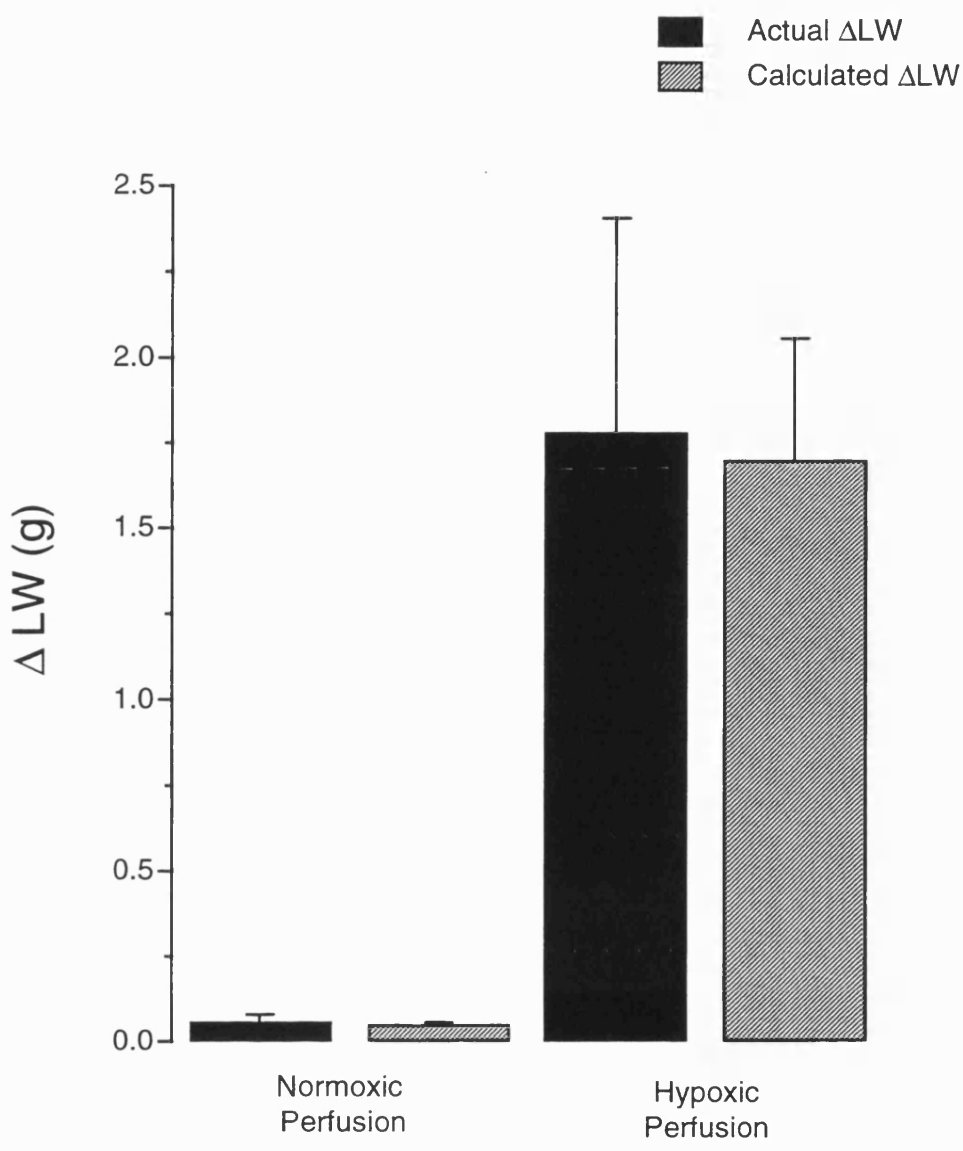


Figure 3.12. Actual changes in lung weight and back calculated fluid accumulation from Evans blue dye in Normoxic and Hypoxic isolated perfused rat lungs. n=5-6.

RECIRCULATING PERFUSION

Following the demonstration of the involvement of endothelins in a single pass system, a recirculating system was devised from which the perfusate could be analysed for endothelin content. The experimental conditions for the single pass model were utilised and some of the perfusate samples collected and stored.

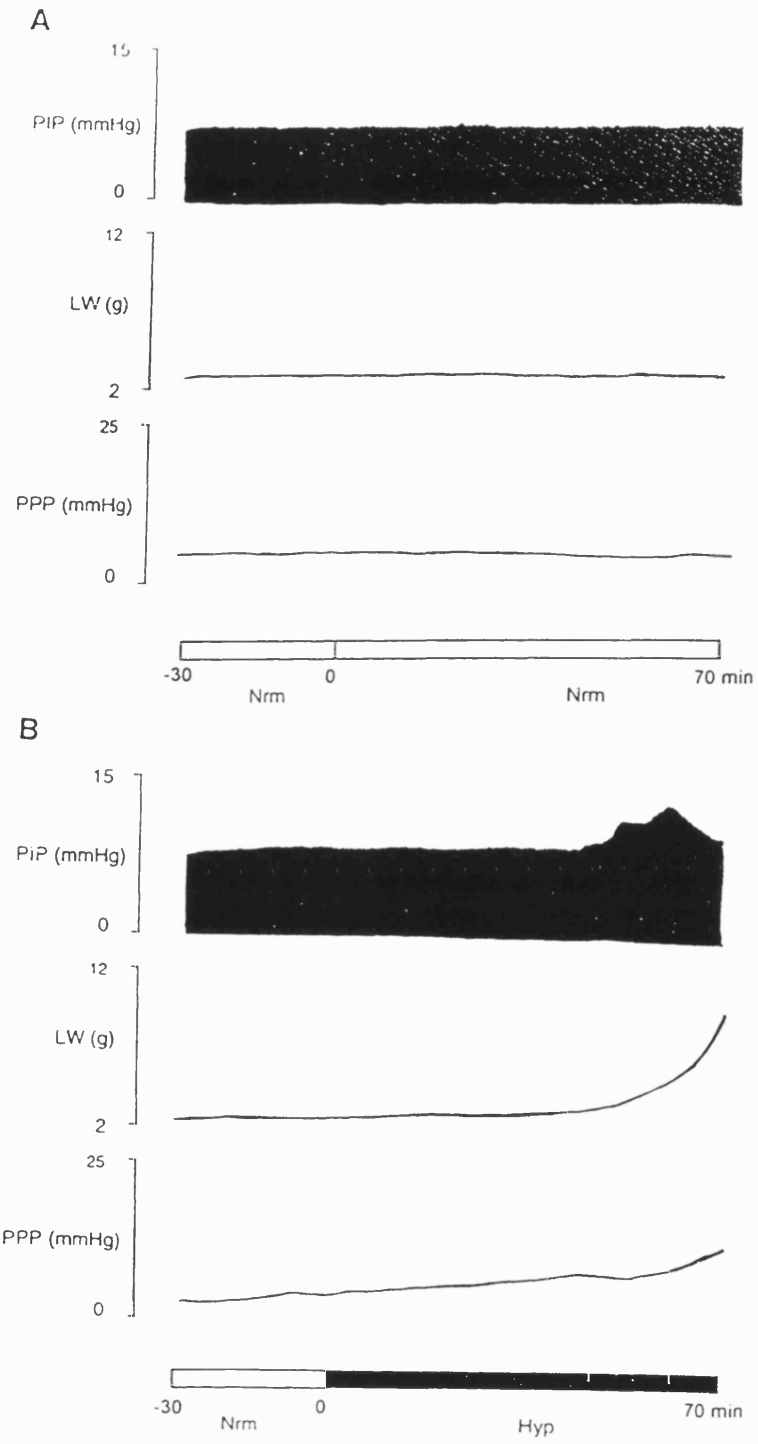
Basal Parameters

Following the 15 min stabilisation period, PPP was 6.8 ± 0.17 mmHg and the increase in LW was 0.057 ± 0.007 g (n=60).

Effects of Hypoxia

Recirculating normoxic perfusion caused a fall in PPP from 7.4 ± 0.7 mmHg to 7.1 ± 0.7 mmHg (n=7) following 85 min recirculating perfusion (see Figure 3.13). LW increased by 0.17 ± 0.05 g over the same period (n=7). Recirculation of a hypoxic perfusate resulted in an increase in PPP from 8.6 ± 0.6 mmHg to 11.4 ± 0.7 mmHg (n=7, $p<0.05$). This was accompanied by an increase in LW of 1.7 ± 0.7g (n=7, $p<0.001$, see Figure 3.14). The increase in vascular resistance was slow to develop and did not reach a plateau during the course of the experiment. LW began to increase after approximately 40 min hypoxia. As with the single pass experiments, there was no significant

difference in PIP in either normoxic or hypoxic lungs, therefore the data has been omitted.


A
15
PIP (mmHg)
0
12
LW (g)
2
25
PPP (mmHg)
0
-30
0
70 min
Nrm
Nrm
B
15
PIP (mmHg)
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12
LW (g)
2
25
PPP (mmHg)
0
-30
0
70 min
Nrm
Hyp

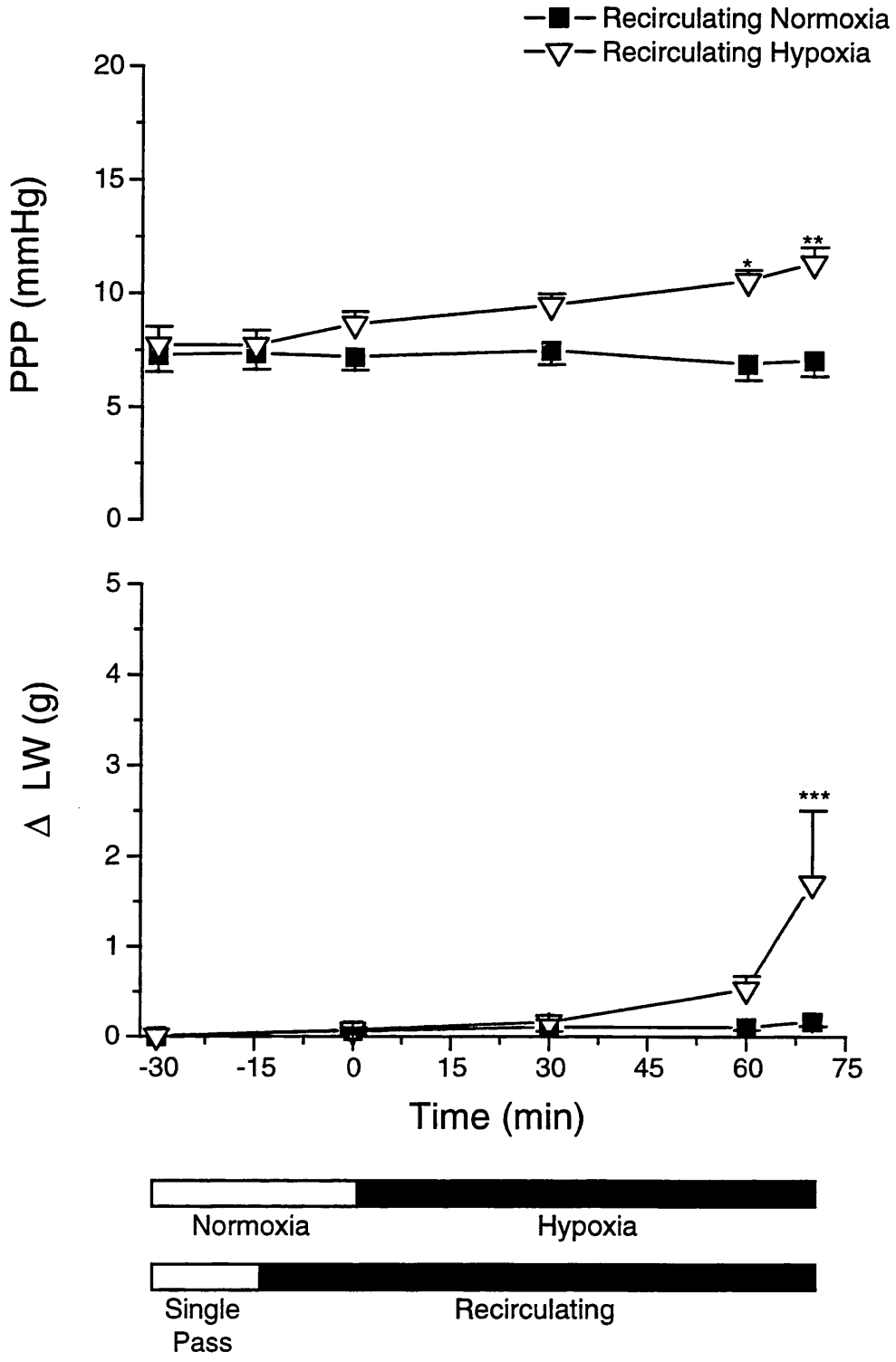

Figure 3.14. The effect of Recirculating Normoxic and Hypoxic perfusion on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in the isolated ventilated perfused rat lung. n=7, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs time-matched normoxic controls.

EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISTS ON RESPONSES TO HYPOXIA

(a) BQ123

Addition of the endothelin ET_A receptor antagonists BQ123 (10 μM) to the recirculating perfusate attenuated the hypoxia-induced increase in PPP from 11.4 ± 0.7 mmHg to 8.5 ± 0.4 mmHg (n=3-7, $p<0.05$; see Figure 3.15). In addition, BQ123 also reduced the rise in LW caused by hypoxic perfusion from 1.7 ± 0.7 g to 0.5 ± 0.01 g (n=3-7, $p<0.05$).

(b) BQ788

The endothelin ET_B receptor antagonist BQ788 (3μM) caused a significant reduction in both the hypoxia-induced increases in vascular resistance and lung weight from 11.4 ± 0.7 mmHg and 1.7 ± 0.7 g to 7.8 ± 0.3 mmHg and 0.3 ± 0.1 g respectively (n=4-7, $p<0.05$ in both cases; see Figure 3.16)

(c) Bosentan

The mixed ET_A/ET_B receptor antagonist bosentan (1.5μM) attenuated the hypoxia-evoked increases in PPP and LW, from 11.4 ± 0.7 mmHg to 8.1 ± 0.7 (n=4, $p<0.05$). In addition to this the hypoxia-induced increase in LW was also reduced by bosentan from 1.7 ± 0.7 g to 0.3 ± 0.1 (n=4, $p<0.01$; see Figure 3.17).

EFFECTS OF AN ENDOTHELIN CONVERTING ENZYME INHIBITOR ON RESPONSE TO HYPOXIA

Phosphoramidon

Following 85 min recirculating perfusion in the presence of 1 μM phosphoramidon PPP was 7.6 ± 1.0 mmHg (n=6) vs 11.4 ± 0.7 mmHg, (n=7). In addition to this the increase in LW in the presence of 1 μM phosphoramidon was 2.8 ± 0.9 (n=6), when compared to the time-matched hypoxic control value of 1.7 ± 0.7g (n=7, see Figure 3.18).

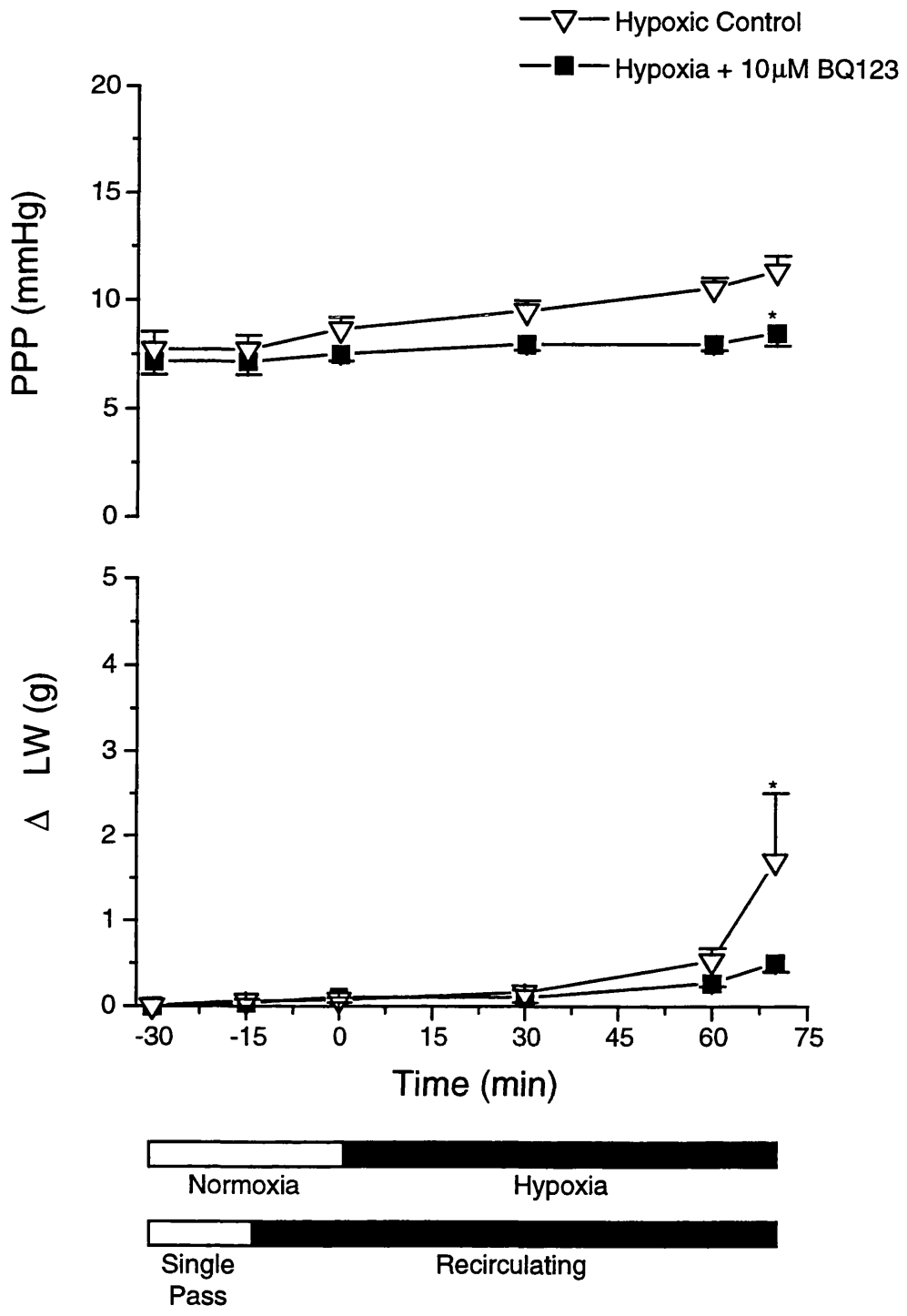

Figure 3.15. The effect of BQ123 (10μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=6-7, * $p<0.05$ vs hypoxic control.

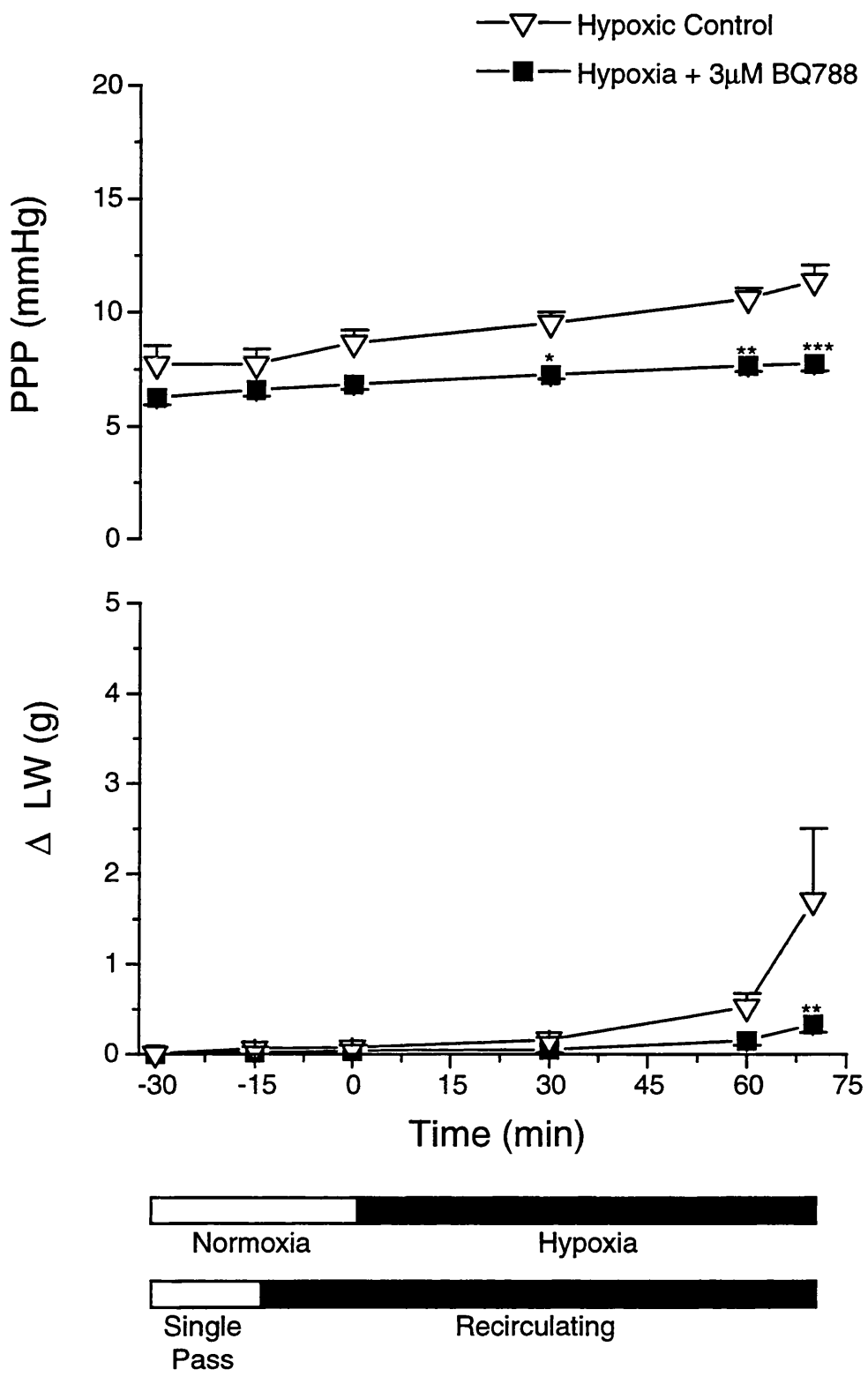

Figure 3.16. The effect of BQ788 (3μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=6-7, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs hypoxic control.

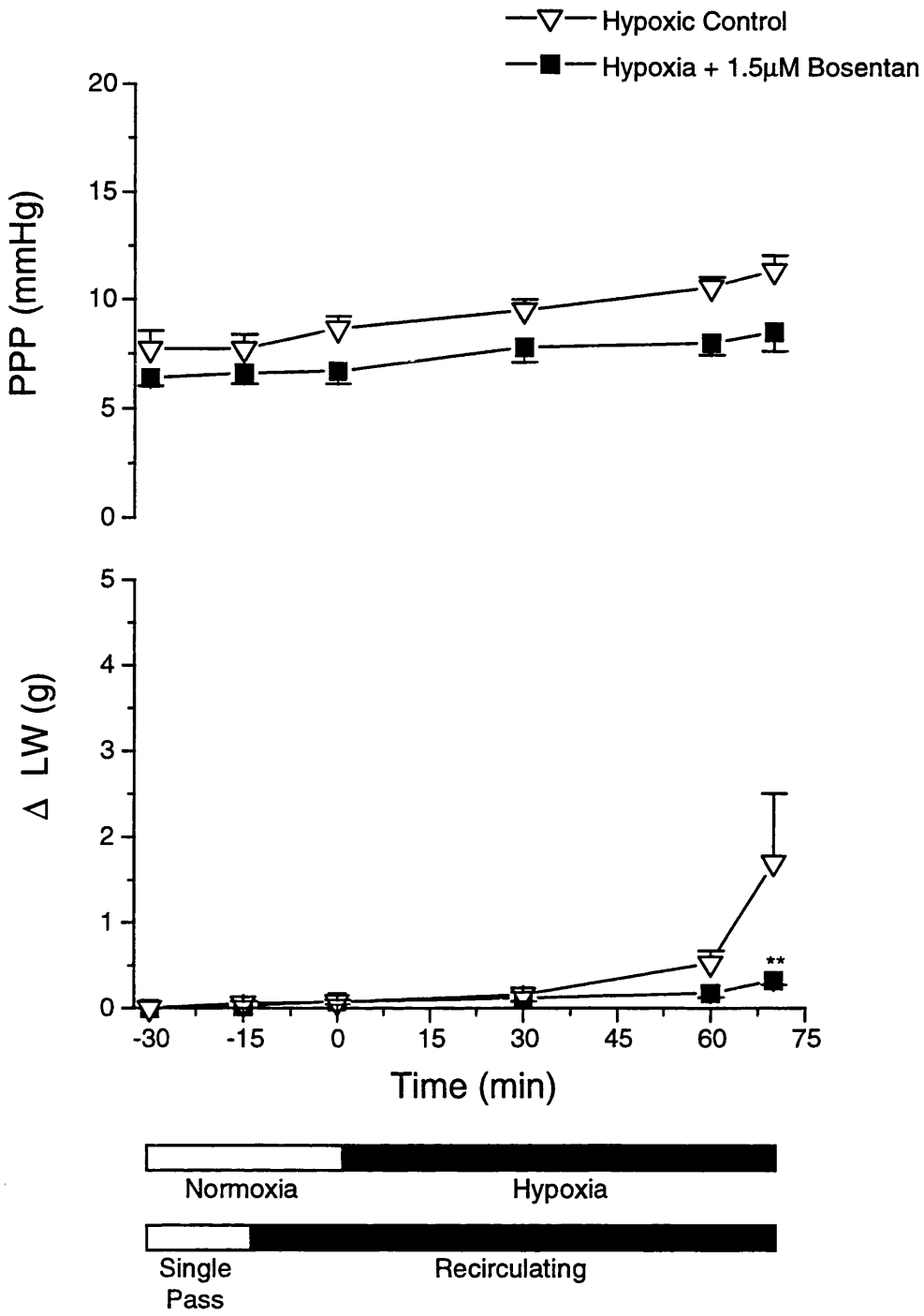

Figure 3.17. The effect of Bosentan (1.5μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=5-7, ** $p<0.01$ vs hypoxic control.

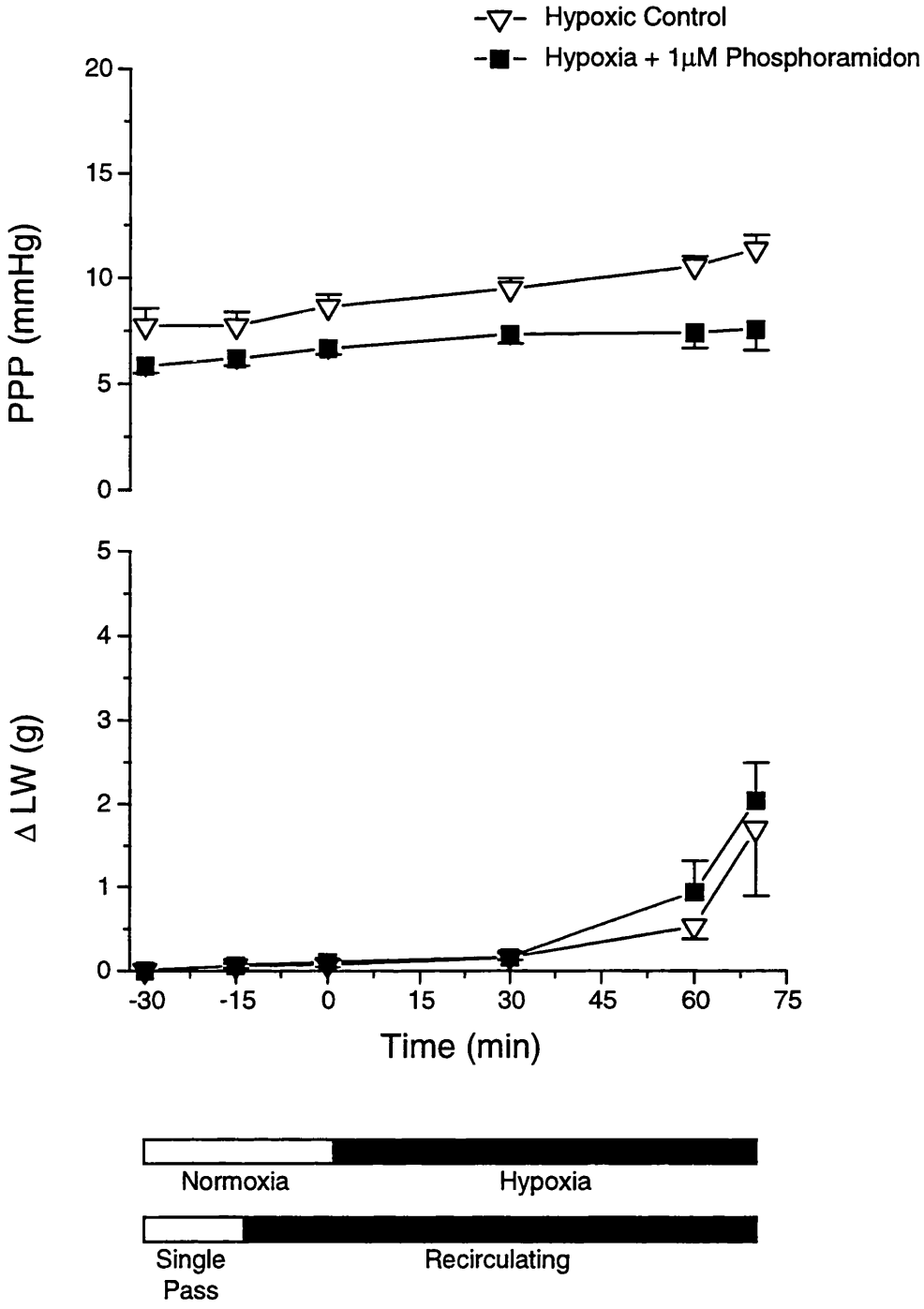

Figure 3.18. The effect of Phosphoramidon (1μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=6-7.

EFFECTS OF CYTOSKELETAL MODIFYING AGENTS ON RESPONSES TO HYPOXIA

Phalloidin and colchicine

When compared to time-matched hypoxic controls, the f-actin stabiliser phalloidin (50 nM) and the microtubule disrupting agent colchicine (100 nM) attenuated the hypoxia-induced increase in PPP from 11.4 ± 0.7 mmHg (n=7) to 7.4 ± 0.4 mmHg (n=4, $p<0.01$) and 8.8 ± 0.8 mmHg (n=5, $p<0.05$) respectively. Both the phalloidin and the colchicine significantly attenuated the hypoxia induced increase in LW from 1.7 ± 0.7g to 0.3 ± 0.08 mmHg and 0.3 ± 0.07 mmHg respectively ($p<0.01$, n=4-7, see Figures 3.19 and 3.20).

EFFECTS OF A PEPTIDE SYNTHESIS INHIBITOR ON THE RESPONSES TO HYPOXIA

Cycloheximide.

The protein synthesis inhibitor cycloheximide (5 µM) significantly reduced the hypoxia induced increase in both PPP (7.0 ± 0.4 mmHg, $p<0.001$, n=4) and LW (0.3 ± 0.1 g, $p<0.05$, n=4) when compared to time-matched controls (11.4 ± 0.7 mmHg, n=7 and 1.7 ± 0.7g, n=7 respectively, see Figure 3.21).

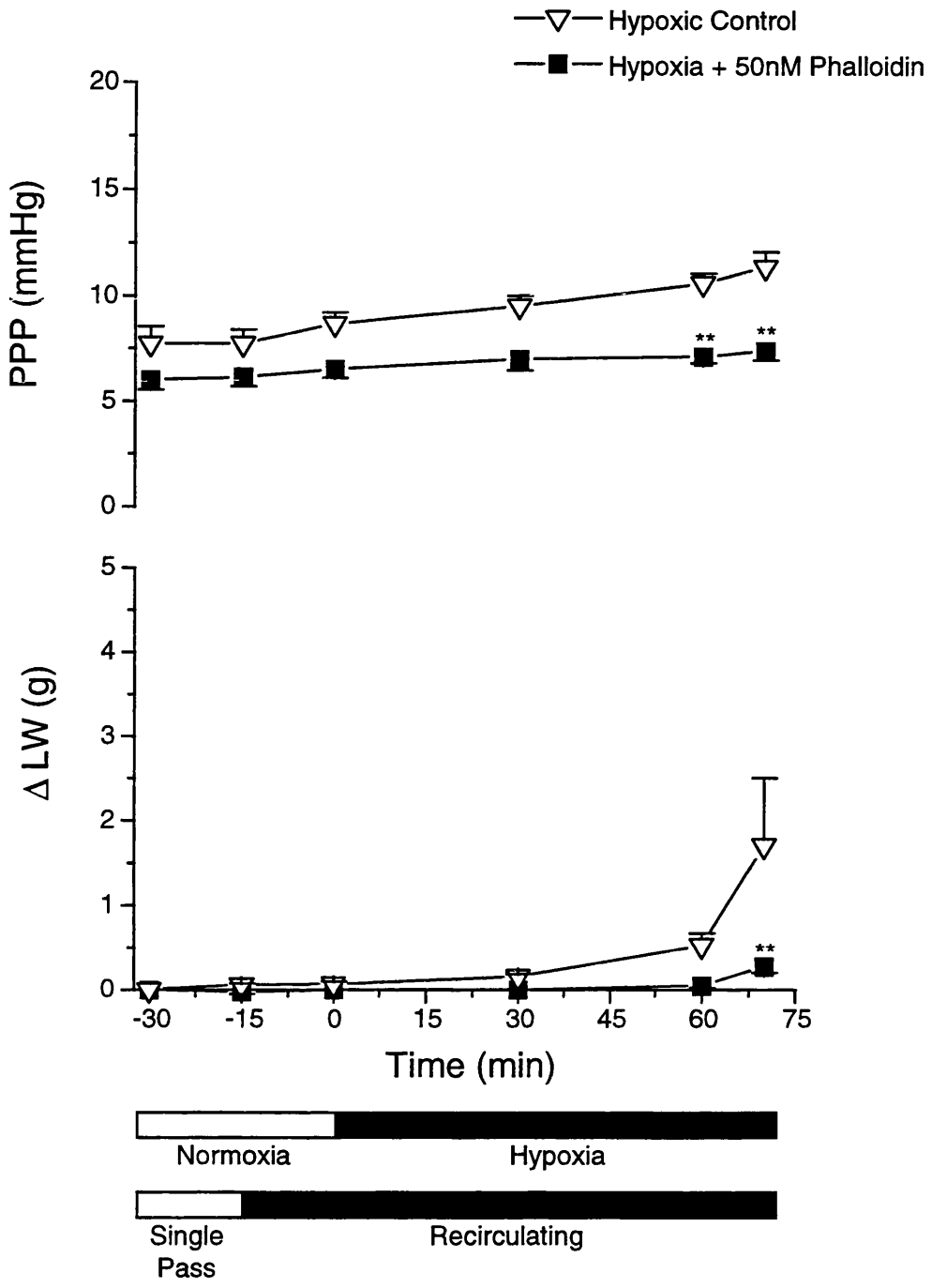

Figure 3.19. The effect of Phalloidin (50 nM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=4-7, ** $p<0.01$ vs time-matched hypoxic control.

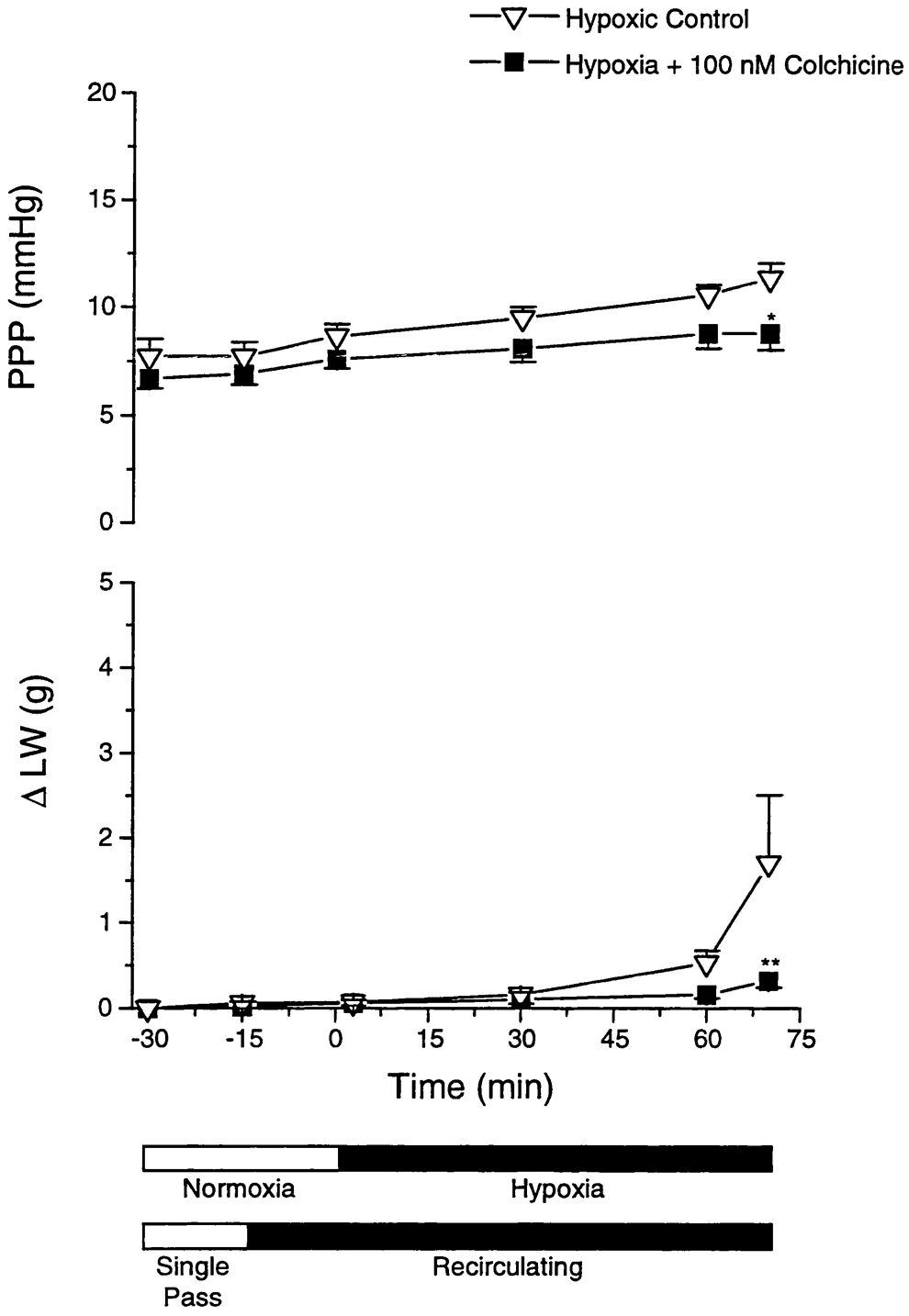

Figure 3.20. The effect of Colchicine (100nM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=5-7, * $p<0.05$; ** $p<0.01$ vs time-matched hypoxic control.

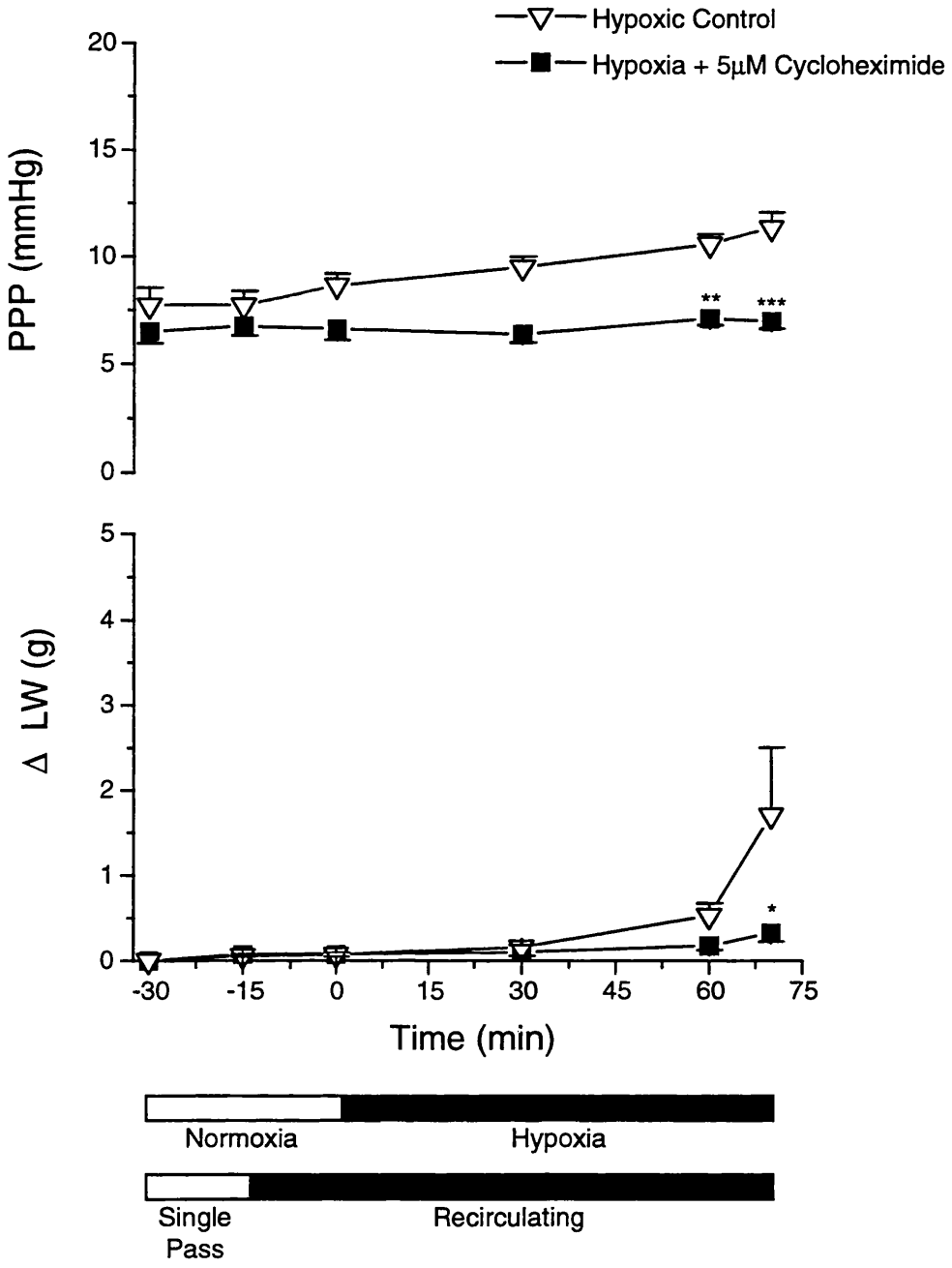

Figure 3.21. The effect of Cycloheximide (5μM) on Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) following hypoxic perfusion in the isolated ventilated recirculating perfused rat lung. n=4-7, * $p<0.05$; ** $p<0.01$;*** $p<0.001$ vs time-matched hypoxic control.

PERFUSATE ENDOTHELIN-1 LEVELS

Following the above recirculating perfusion experiments where PPP, PIP and LW were recorded, the perfusates were collected and stored at -80°C for ET-1 determination.

Effects of Hypoxia

Normoxic perfusion (90 min) resulted in an ET-1 level of 0.23 ± 0.03 pg/ml perfusate (n=9). Following 90 min hypoxic perfusion the ET-1 level had increased to 0.93 ± 0.10 pg/ml perfusate (n=8, $p<0.001$, see Figure 3.22).

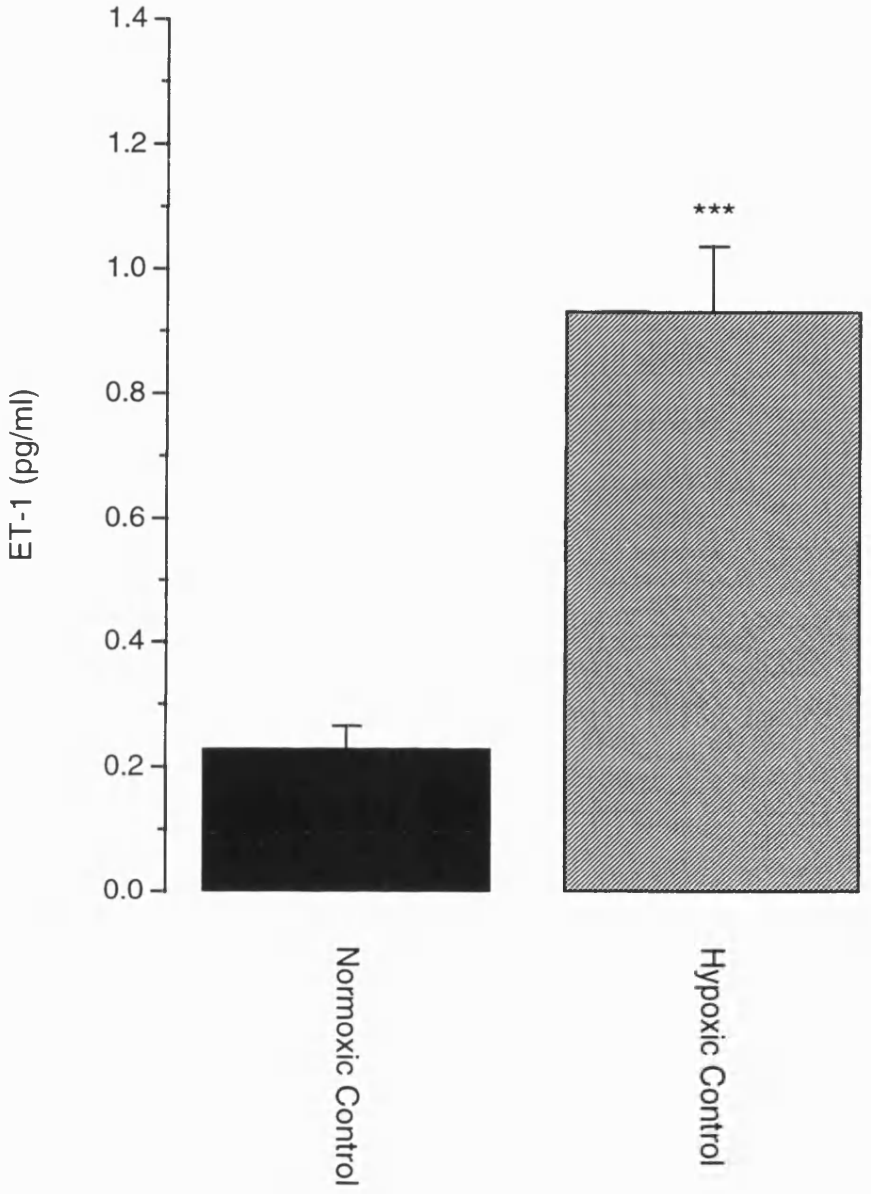

Figure 3.22. The effect of Normoxic and Hypoxic recirculating perfusion in rat lung on perfusate levels of ET-1. n=8-9, *** $p<0.001$.

EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISTS ON ENDOTHELIN-1 LEVELS

(a) BQ123

The endothelin ET_A receptor antagonist BQ123 (1.5 μM) significantly lowered circulating ET-1 levels when compared to time-matched hypoxic controls (0.40 ± 0.10 pg/ml vs 0.93 ± 0.10 pg/ml, n=6-8, $p<0.01$; see Figure 3.23).

(b) BQ788

BQ788 (3 μM), an ET_B receptor antagonist, significantly increased ET-1 levels to 2.62 ± 0.43 pg/ml (n=6, $p<0.01$), when compared to hypoxic controls (0.93 ± 0.10 pg/ml, n=8; see Figure 3.23).

(c) Bosentan

The mixed ET_A/ET_B receptor antagonist bosentan (1.5 μM) attenuated the hypoxia induced increase in ET-1 from 0.93 ± 0.10 pg/ml to 0.35 ± 0.9 pg/ml (n=5-8, $p<0.01$; see Figure 3.23).

EFFECT OF AN ENDOTHELIN CONVERTING ENZYME INHIBITOR ON ENDOTHELIN-1 LEVELS

Phosphoramidon

The endothelin converting enzyme inhibitor phosphoramidon (1μM) significantly reduced the ET-1 levels when compared to the hypoxic control and significantly increased the levels when compared to the normoxic control (0.58 ± 0.1 pg/ml vs 0.93 ± 0.10 pg/ml and 0.23 ± 0.03 pg/ml respectively, n=3-9, $p<0.05$; see 3.23).

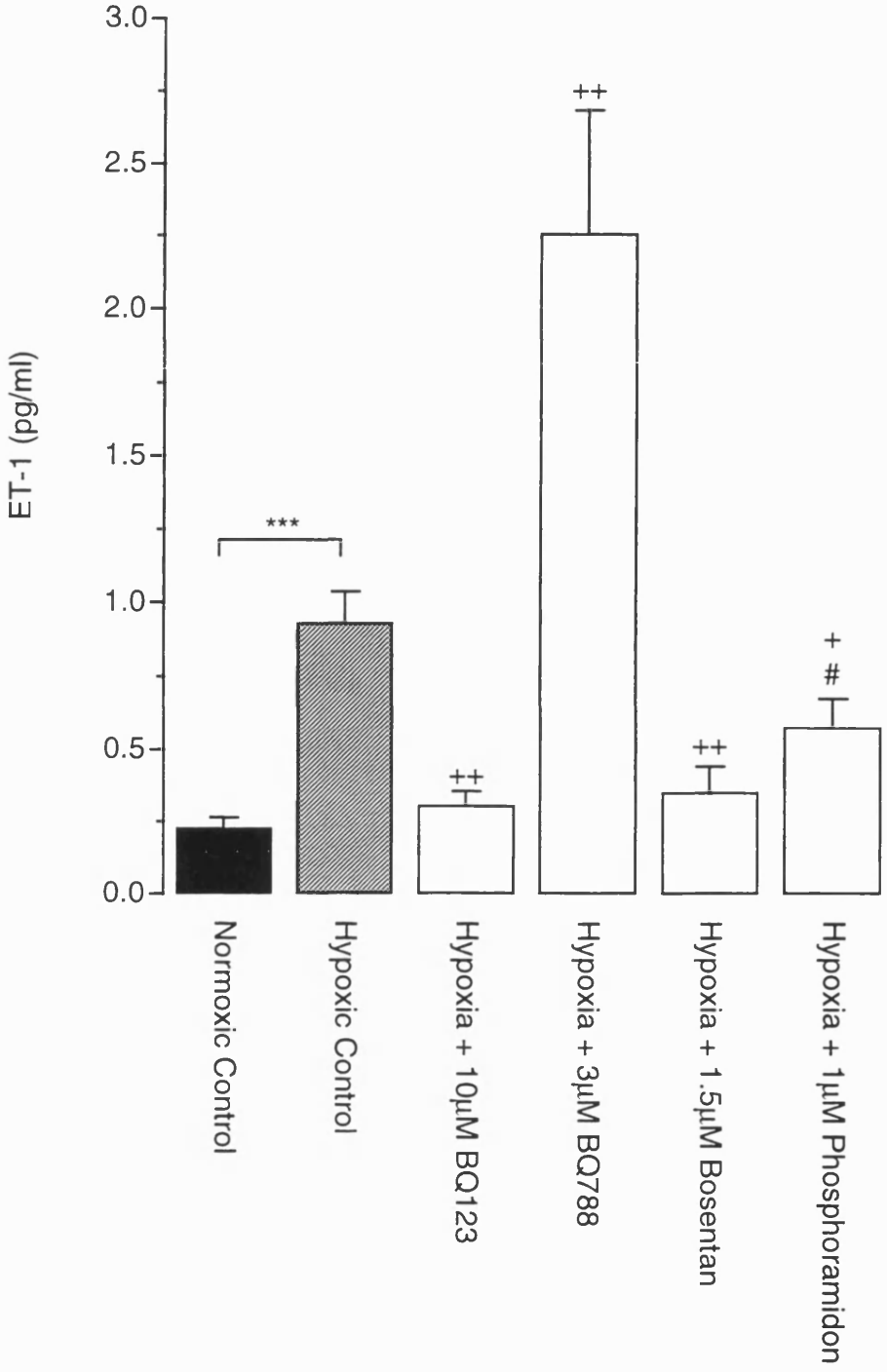

Figure 3.23. The effect of BQ123, BQ788, bosentan and phosphoramidon on perfusate ET-1 levels following recirculating hypoxic perfusion in rat lung. *** $p<0.001$ vs Normoxic control; + $p<0.05$, ++ $p<0.01$ vs Hypoxic control; # $p<0.05$ vs Normoxic control, n=3-9.

EFFECT OF CYTOSKELETAL MODIYFYING AGENTS ON ENDOTHELIN-1 LEVELS

Phalloidin and Colchicine

The f-actin stabiliser phalloidin (50 nM) and the microtubule disrupting agent colchicine (100nM) significantly reduced the circulating ET-1 levels when compared to hypoxic time-matched controls (0.32 ± 0.07 pg/ml and 0.20 ± 0.03 pg/ml respectively vs 0.93 ± 0.10 pg/ml, n=4-8, $p<0.01$; see Figure 3.24).

EFFECT OF A PEPTIDE SYNTHESIS INHIBITOR ON ENDOTHELIN-1 LEVELS

Cycloheximide

The protein synthesis inhibitor cycloheximide (5μM) significantly reduced the hypoxia induced increase in circulating ET-1 levels form 0.93 ± 0.10 pg/ml to 0.12 ± 0.04 pg/ml, n=5-8, $p<0.001$ (see Figure 3.24).

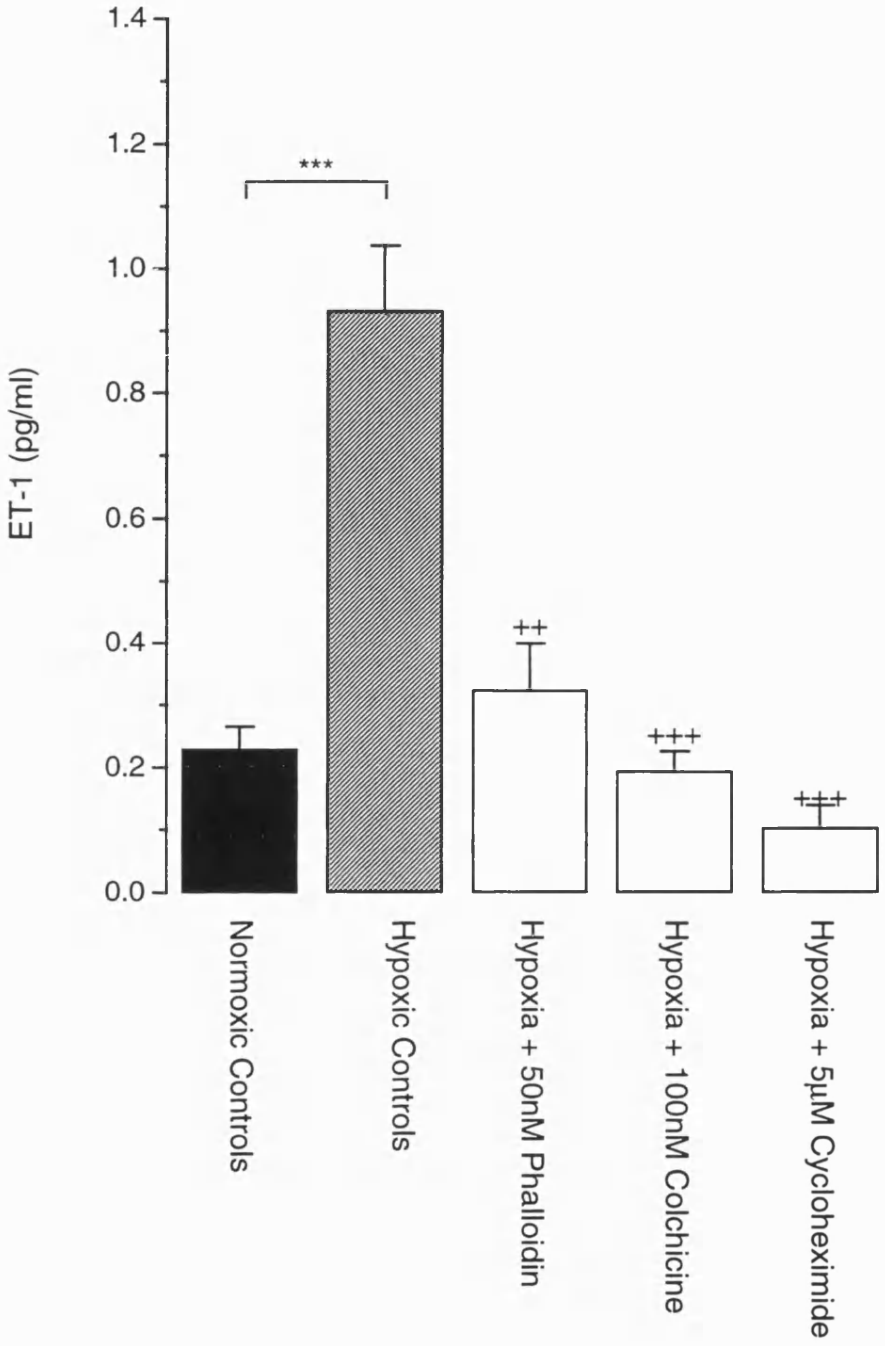

Figure 3.24. The effect of Phalloidin, Colchicine and Cycloheximide on perfusate ET-1 levels following recirculating hypoxic perfusion in rat lung. *** $p<0.001$ vs Normoxic control; +++ $p<0.001$, ++ $p<0.01$ vs Hypoxic control, n=4-9.

TISSUE ENDOTHELIN-1 LEVELS

Exposure to hypoxia

Following 90 min normoxic recirculating perfusion, tissue levels of ET-1 were found to be 141.0 ± 11.1 pg/mg protein (n=6). Recirculating hypoxic perfusion (90 min) resulted in measured tissue levels of ET-1 of 148.5 ± 12.9 pg/mg protein (n=5). This was not significantly different form the normoxic tissue level (see Figure 3.25).

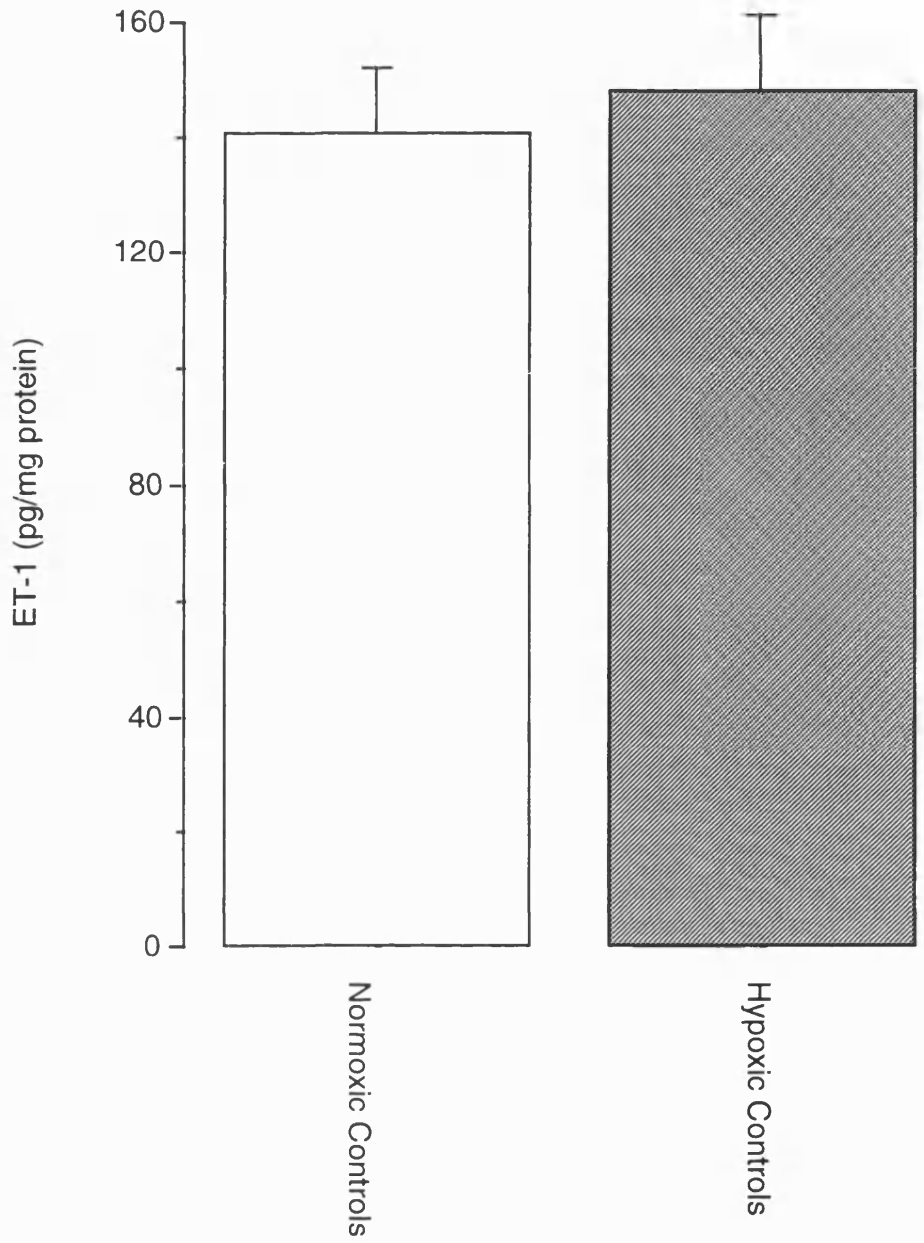

Figure 3.25. The effect Normoxic and Hypoxic recirculating perfusion on tissue ET-1 levels in rat lung, n=5-6.

EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISTS ON TISSUE ENDOTHELIN-1 LEVELS

(a) BQ123

The endothelin ET_A receptor antagonist BQ123 (10 μM) lowered the tissue levels of ET-1 to 113.8 ± 16.0 pg/mg protein (n=4) when compared to the hypoxic control (148.5 ± 12.9 pg/mg protein, n=5), however this was not significant (see Figure 3.26).

(b) Bosentan

The mixed ET_A/ET_B receptor antagonist bosentan (1.5 μM) significantly lowered the tissue ET-1 level to 73.2 ± 7.9 pg/mg protein (n=5) when compared to both the normoxic and hypoxic controls (141.0 ± 11.1 pg/mg protein, n=6, $p<0.05$ and 148.5 ± 12.9 pg/mg protein, n=5, $p<0.01$; see Figure 3.27).

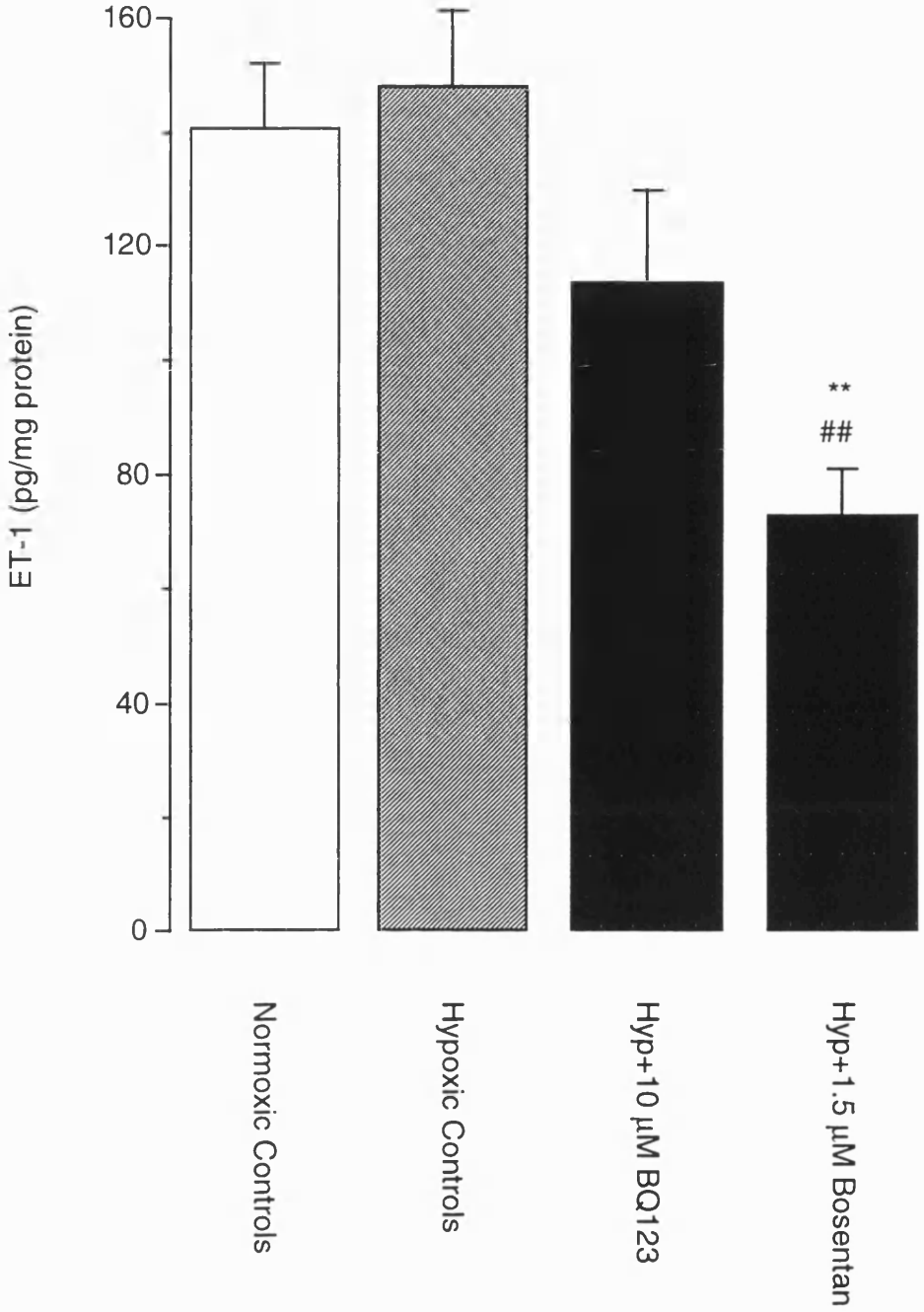

Figure 3.26. The effect of BQ123 and Bosentan on tissue ET-1 levels following recirculating hypoxic perfusion in rat lung, n=4-6; ## $p < 0.01$ vs Normoxic; ** $p < 0.01$ vs Hypoxic.

EFFECT OF CYTOSKELETAL MODIYFYING AGENTS ON TISSUE ENDOTHELIN-1 LEVELS

Phalloidin and Colchicine

Both the f-actin stabiliser phalloidin (50 nM) and the microtubule disrupting agent colchicine (100 nM) reduced the hypoxic tissue ET-1 levels from 148.5 ± 12.9 pg/mg protein to 115.5 ± 9.1 and 117.4 ± 17.1 pg/mg protein (n=4-5). However these differences were not significant (see Figure 3.27).

EFFECT OF A PEPTIDE SYNTHESIS INHIBITOR ON TISSUE ENDOTHELIN-1 LEVELS

Cycloheximide

The peptide synthesis inhibitor cycloheximide (5 μM) significantly reduced the tissue ET-1 levels when compared to both the normoxic and hypoxic controls (79.0 ± 8.7 pg/mg protein vs 141.0 ± 11.1 and 148.5 ± 12.9 pg/mg protein (n=4-6; $p < 0.05$; see Figure 3.27).

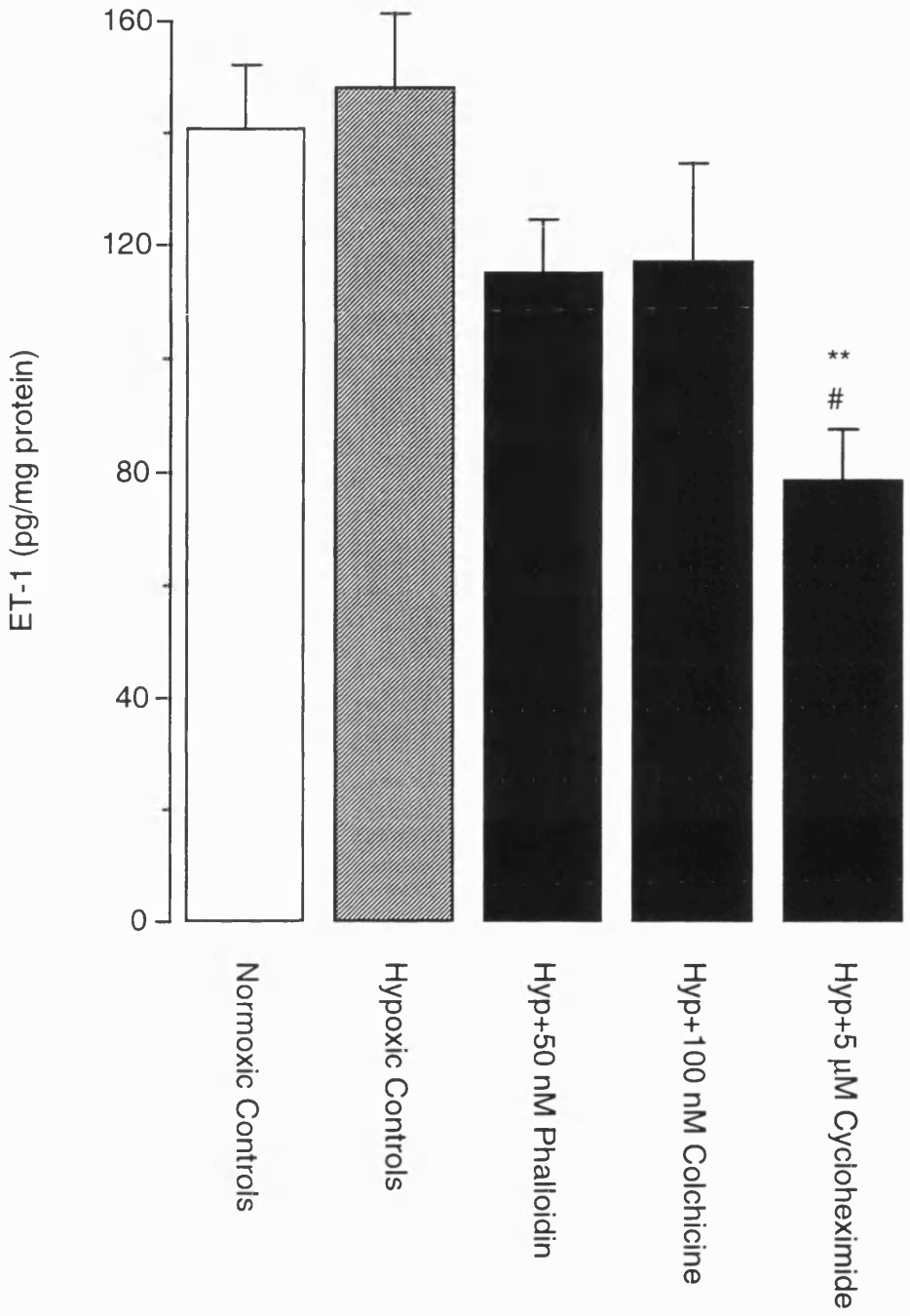

Figure 3.27. The effect of Phalloidin, Colchicine and Cycloheximide on tissue ET-1 levels following recirculating hypoxic perfusion in rat lung, n=4-6; # $p < 0.05$ vs Normoxic; ** $p < 0.01$ vs Hypoxic.

TISSUE PREPRO ENDOTHELIN-1 mRNA LEVELS

The results from the PCR amplification of the prepro ET-1 mRNA show an increase in tissue levels following 90 min perfusion of single pass hypoxia, when observed against time-matched normoxic tissues (see Figure 3.28). The results for both the prepro endothelin-1 and rat β-actin are taken from a sub-maximal amplification cycle.

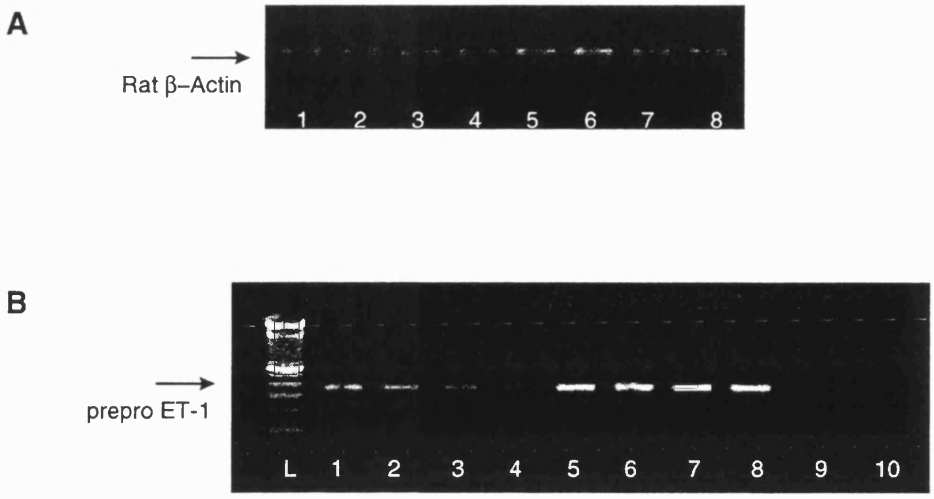

Figure 3.28. The effect of 90 min single pass hypoxic perfusion on A) rat ß-actin and B) rat prepro ET-1 mRNA levels in the isolated rat lung. L 100bp ladder; 1-4 Normoxic perfusion; 5-8 Hypoxic perfusion; 9 sterile distilled water control; 10 running buffer control.

The bolus dose used to illustrate the effects of ETs and Sx6c on the measured parameters, was picked in response to its actions on the preparation. The dose produces a sub-maximal response which is significant when compared to basal parameters.

Endothelin-1

The effects of endothelin-1 (ET-1) on PPP, PIP and LW are illustrated in Figure 3.29. It can be seen that ET-1 caused a dose-dependent increase in PPP, PIP and LW (50 - 800 pmol). The increase in PPP was sustained at low doses (50 - 200 pmol), however, at higher doses (400 - 800 pmol) the increase in PPP was bi-phasic in nature, with a large, rapid, transient rise, which subsided to give a sustained increase. ET-1 (400 pmol) increased PPP by 11.5 ± 0.74 mmHg (n=8).

ET-1 caused a steep and irreversible dose-dependent increase in LW (see Figures 3.29 and 3.33), which was associated with the appearance of fluid in the tracheal cannula, with a 1.04 ± 0.35 g (n=8) increase with a 400pmol dose.

In addition to this, ET-1 also caused a dose-dependent increase in PIP (see Figures 3.29 and 3.33) . A 400 pmol bolus dose increased PIP by 2.89 ± 0.29 mmHg (n=8).

Endothelin-3

As can be seen in Figure 3.30 ET-3 caused a dose-dependent increase in PPP, which was sustained at low doses (50 - 400 pmol), but underwent a slow reversal at higher doses (800 - 1600 pmol). The increase in PPP with a 400 pmol bolus dose was 4.25 ± 0.59 mmHg (n=6).

ET-3 also caused a dose-dependent increase in PIP, which was similar to the responses seen with ET-1 (3.42 ± 0.35 mmHg, n=6; see Figures 3.30 and 3.33).

The ET-3 induced increase in LW was irreversible, with a 0.23 ± 0.06 g increase with a 400 pmol bolus dose (see Figures 3.30 and 3.33).

Sarafotoxin 6c

Sarafotoxin 6c (Sx6c) induced dose-dependent increases in PPP, which slowly reversed at both low (50 -200 pmol) and high doses (400 - 800 pmol; see Figures 3.31 and 3.33). 400 pmol Sx6c increased PPP by 8.17 ± 0.59 mmHg (n=5).

In addition to this, Sx6c increased PIP in a dose-dependent fashion, with the lower doses of peptide (50 - 200 pmol), undergoing a slow reverse, and higher doses (400 - 800 pmol) causing a sustained increase in PIP. The 400 pmol dose of Sx6c caused an increase in PIP of 11.5 ± 0.96 mmHg (see Figures 3.31 and 3.33).

Sx6c also increased LW in a dose-dependent fashion, with 400 pmol causing a 5.32 ± 1.56 g increase in LW (see Figures 3.31 and 3.33)

Big Endothelin-1

Big ET-1, the precursor peptide of ET-1, when given as bolus doses to the lung caused dose-dependent increases in PPP, PIP and LW (see Figure 3.32). The increases in PPP were significantly less than the increases observed with equal doses of ET-1 (for example 400 pmol big ET-1 increased PPP by 5.58 ± 0.42 mmHg, compared to the ET-1 response of 11.5 ± 0.74 mmHg (n=6-8, $p<0.001$). It should be noted that an 800 pmol bolus dose of big ET-1 was needed to produce the same increase in vascular resistance as that seen following the administration of a 200 pmol bolus dose of ET-1.

The increase in PIP observed with big ET-1was equipotent with ET-1, with a 400 pmol bolus dose of big ET-1 inducing a 2.35 ± 0.31 mmHg increase in PIP, compared to a 2.89 ± 0.29 mmHg increase for ET-1 (n=6-8).

The increase in LW following administration of big ET-1 was not as great as that seen following the administration of equivalent doses of ET-1, with 400 pmol big ET-1 increasing LW by 0.32 ± 0.08 g, compared to 1.04 ± 0.35 g for ET-1 (n=6-8, $p<0.01$, see Figure 3.34).

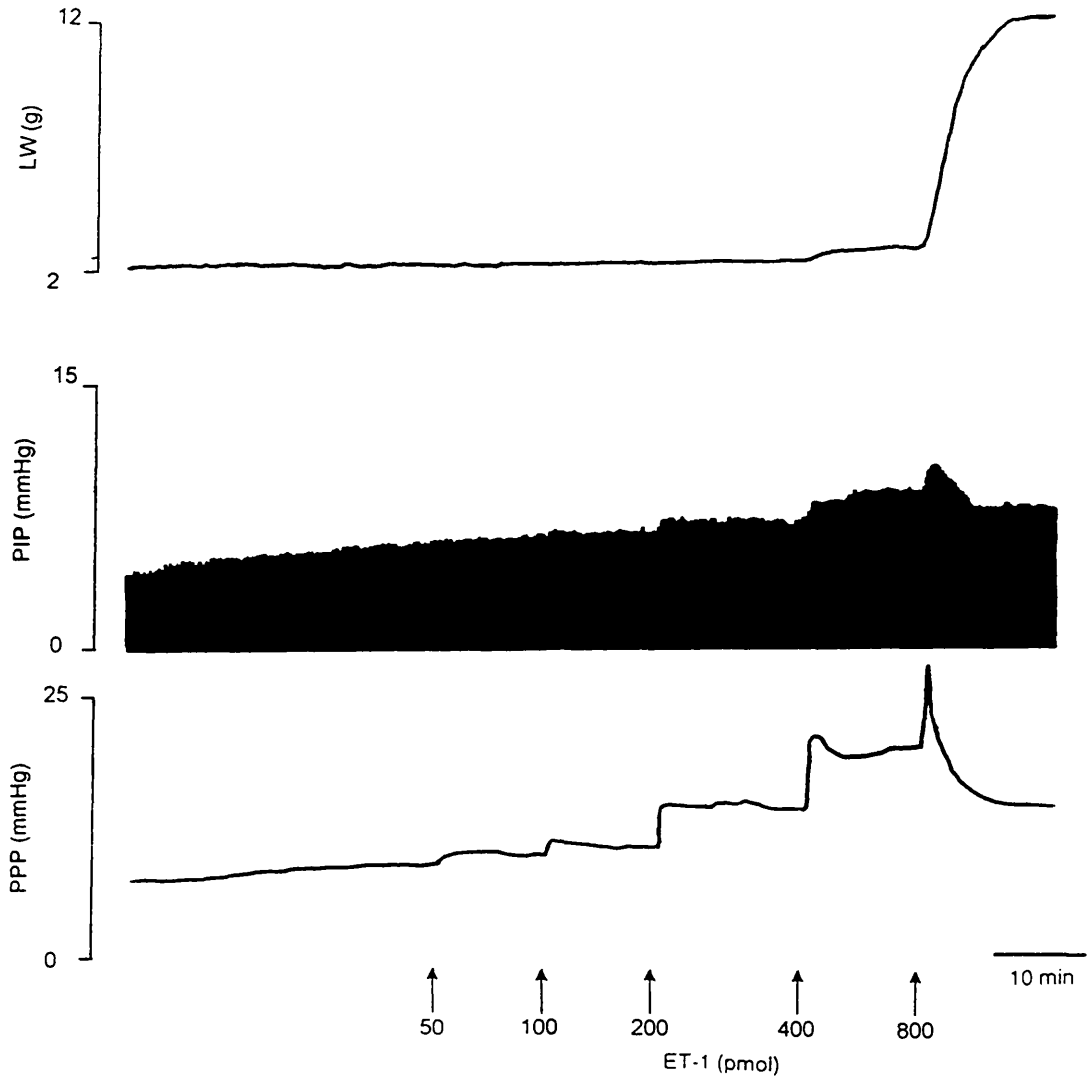

Figure 3.29. The effect of Endothelin-1 on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) in an isolated, ventilated perfused rat lung (representative trace from 8 similar experiments).

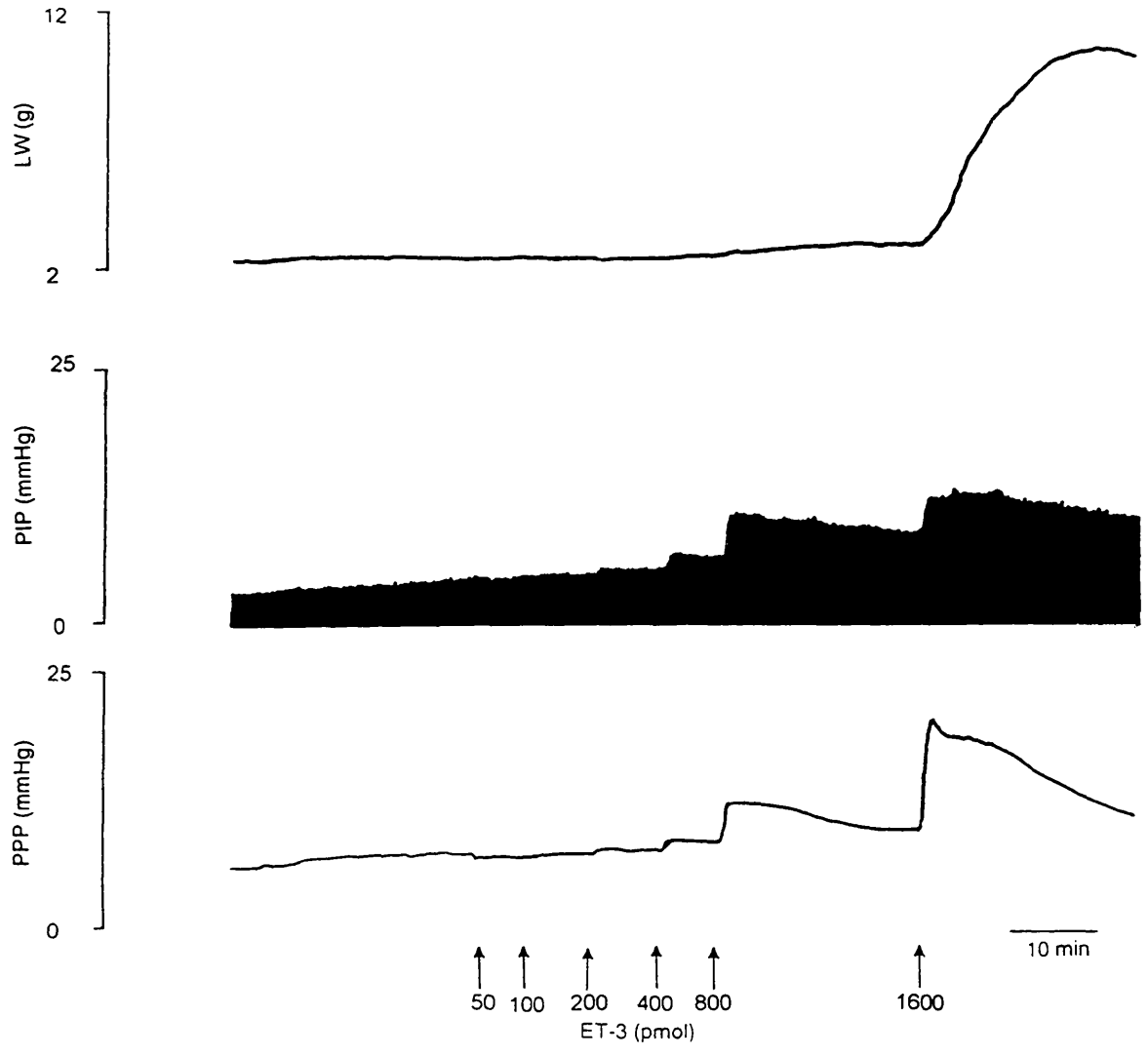

Figure 3.30. The effect of Endothelin-3 on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) in an isolated, ventilated perfused rat lung (representative trace from 6 similar experiments).

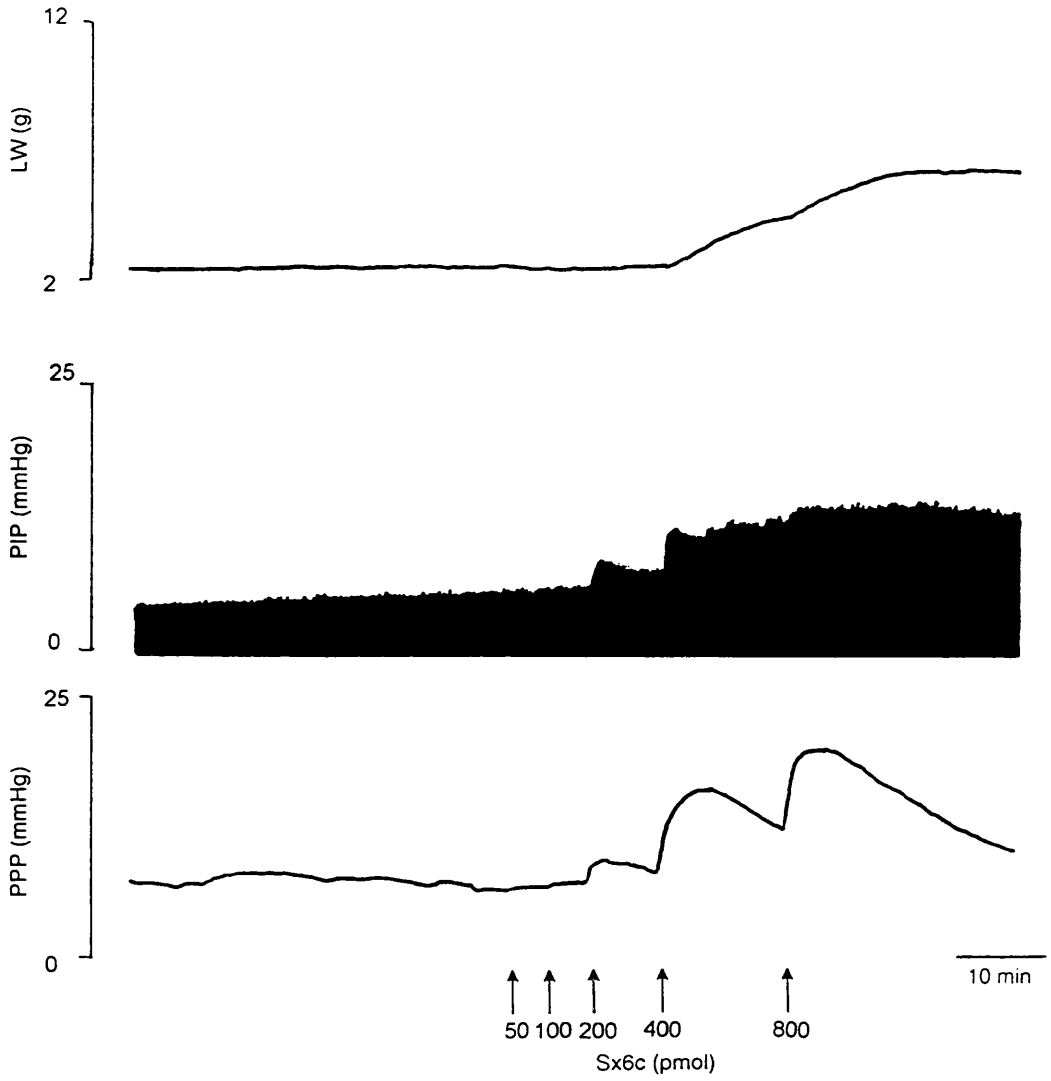

Figure 3.31. The effect of Sarafotoxin 6c on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) in an isolated, ventilated perfused rat lung (representative trace from 5 similar experiments).

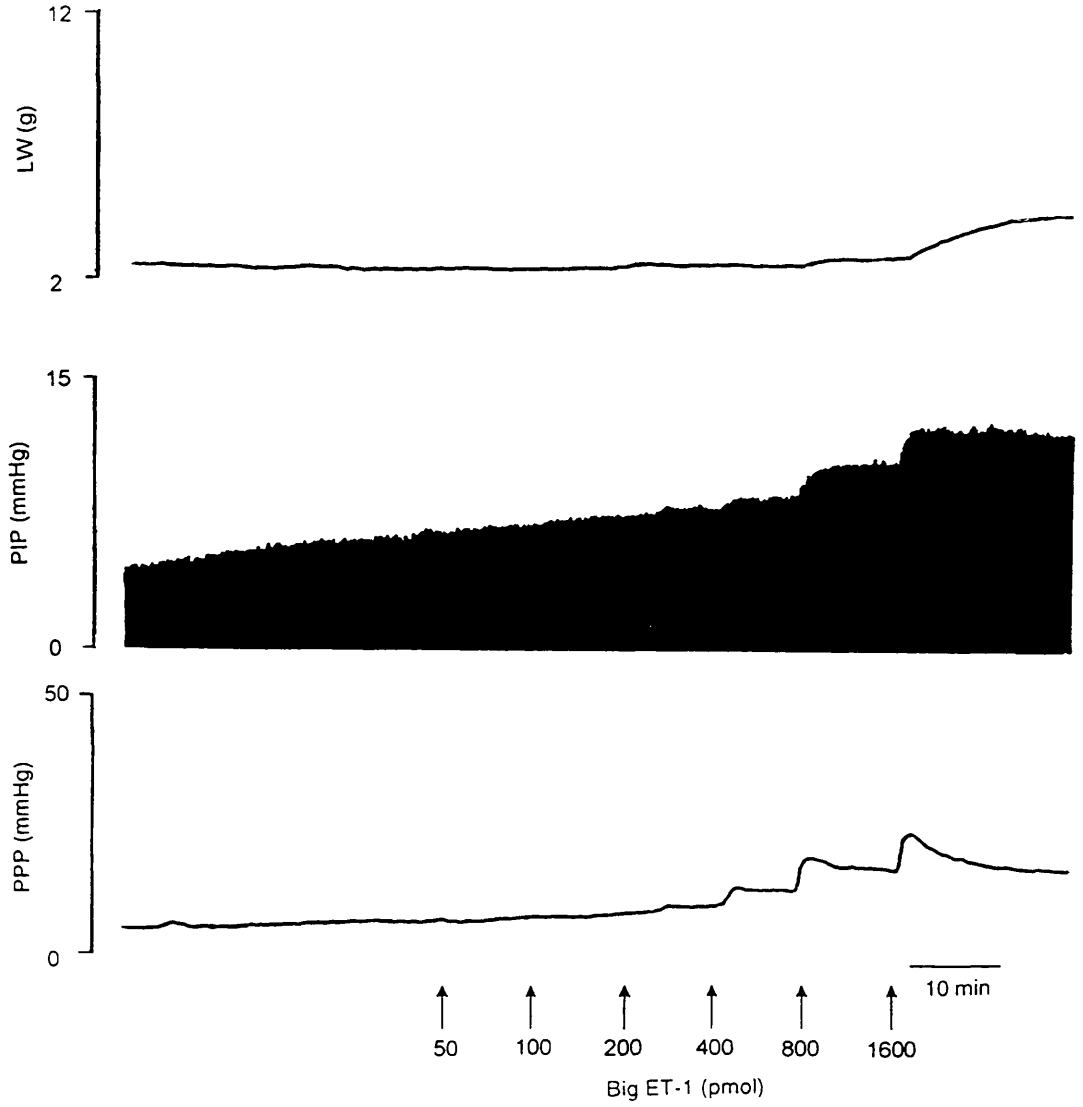

Figure 3.32. The effect of Big Endothelin-1 on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) in an isolated, ventilated perfused rat lung (representative trace from 6 similar experiments).

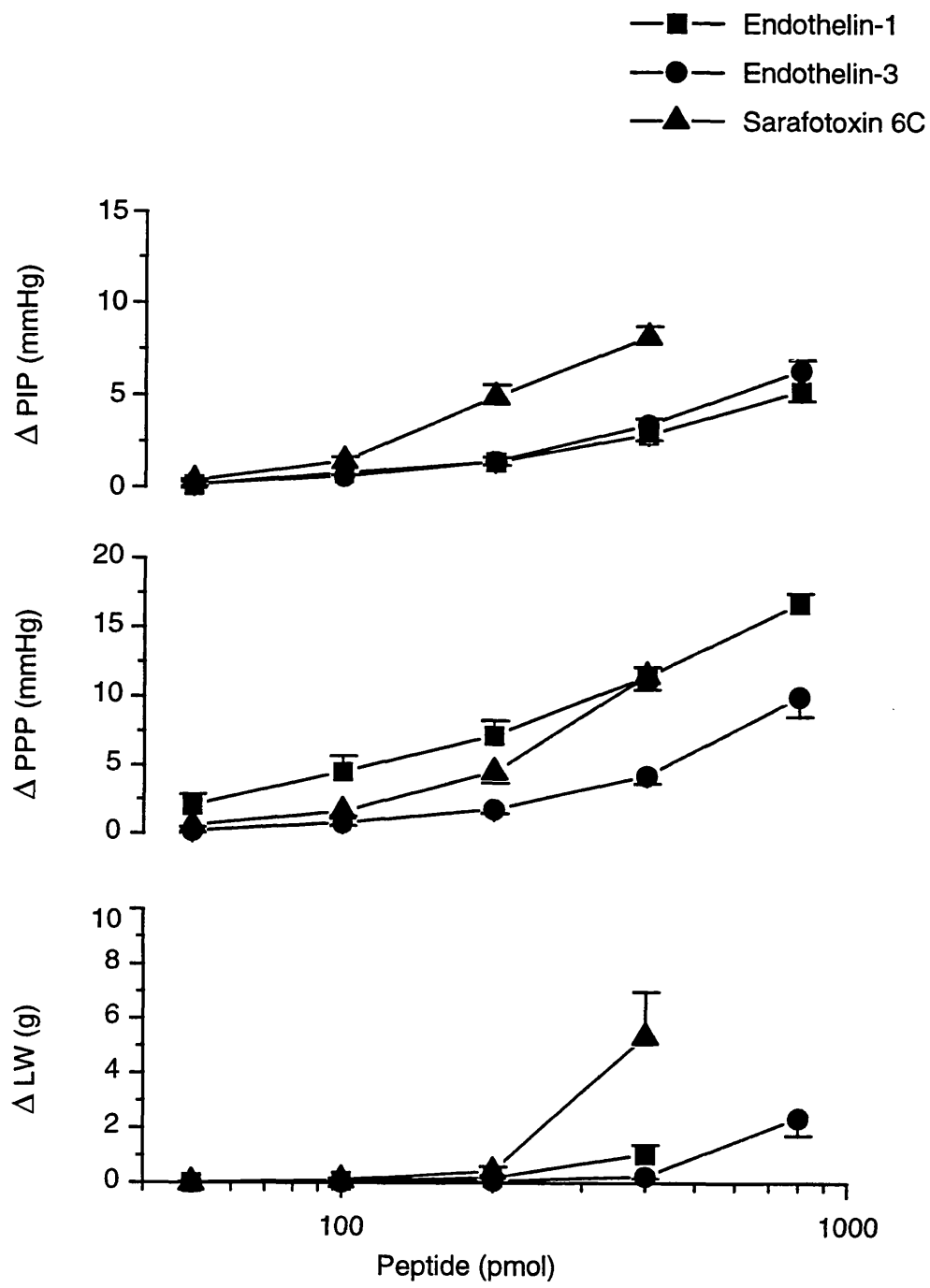

Figure 3.33. The effects of Endothelin-1, Endothelin-3 and Sarafotoxin 6c on changes in Pulmonary Inflation Pressure (PIP), Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in isolated, ventilated perfused rat lungs. n=5-8.

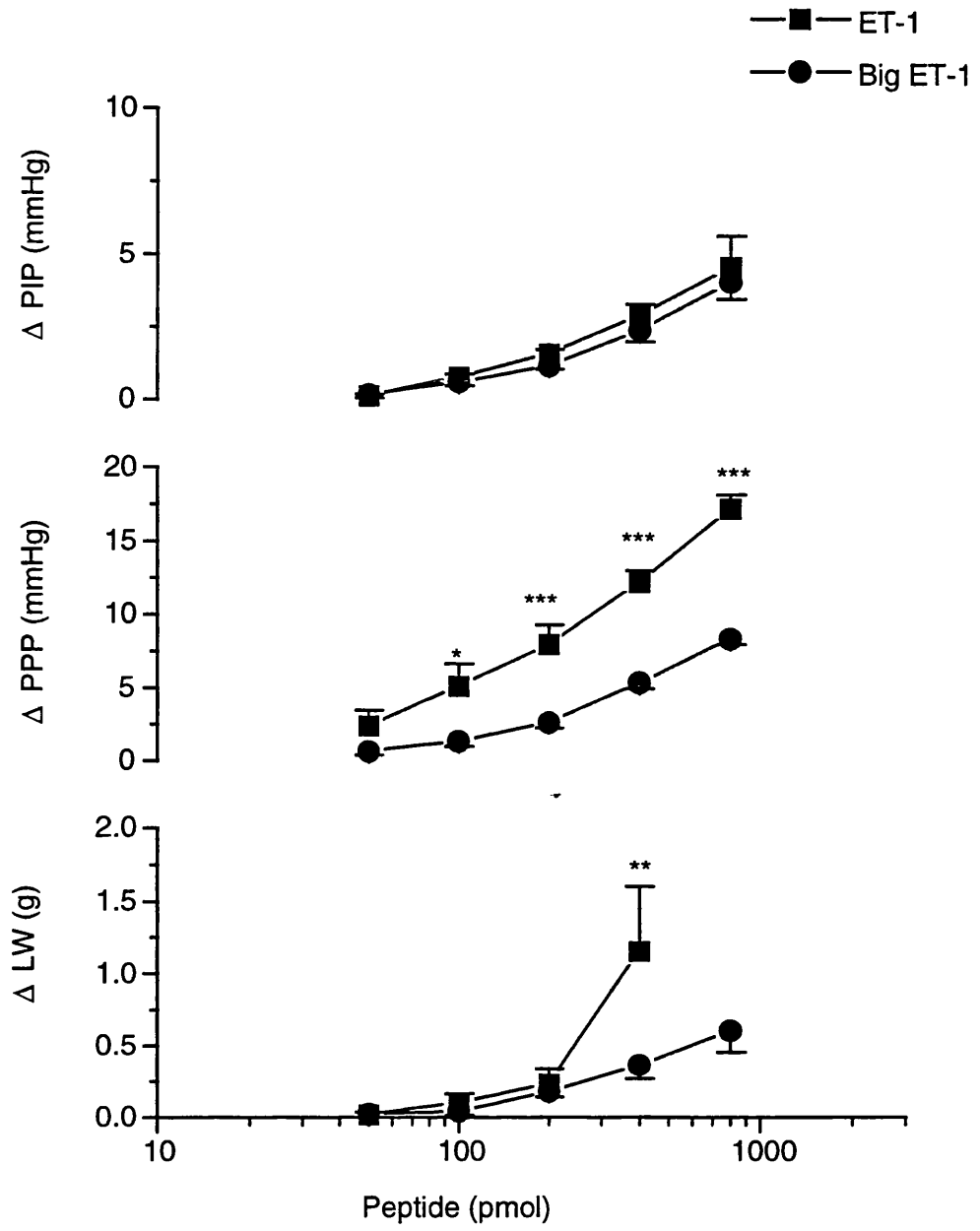

Figure 3.34. The effects of Big Endothelin-1 and Endothelin-1 on changes in Pulmonary Inflation Pressure (PIP), Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in isolated, ventilated perfused rat lungs. n=6-8, * $p<0.05$, ** $P<0.01$, *** $p<0.001$ vs Endothelin-1.

EFFECTS OF ENDOTHELIN RECEPTOR-ANTAGONISTS UPON THE ACTIONS OF ENDOTHELINS AND SARAFOTOXIN ON THE LUNG

Endothelin-1

The endothelin ET_A receptor antagonist BQ123 (3 μM) significantly attenuated the ET-1 induced increase in PPP (for example, 400pmol ET-1 increased PPP by 11.5 ± 0.74 mmHg, whereas in the presence of 3 μM BQ123 the increase in PPP was 2.38 ± 0.38 mmHg, $p<0.001$, n=4-8).

The endothelin ET_B receptor antagonist BQ788 (3 μM) had no effect on the ET-1 induced vasoconstriction, with 400 pmol ET-1 increasing PPP by 10.88 ± 0.80 mmHg (n=4), compared to the control value of 11.5 ± 0.74 mmHg (n=8).

In combination, the ET_A receptor antagonist BQ123 and the ET_B receptor antagonist BQ788 (both 3 μM) significantly inhibited the ET-1 induced increase in PPP (1.5 ± 0.20 mmHg vs 11.5 ± 0.74 mmHg for a 400 pmol bolus dose, n=4-8, $p<0.001$; see Figures 3.35 + 3.39).

BQ123 (3 μM) had no significant effect upon the ET-1 induced increase in PIP, with 400 pmol increasing PIP by 2.38 ± 0.24 in the presence of BQ123, against the ET-1 control of 2.89 ± 0.29 mmHg (n=4-8).

The ET_B receptor antagonist BQ788 (3 μM) significantly increased the ET-1 induced increase in PIP, with 400pmol ET-1 increasing PIP by 4.75 ± 1.20 mmHg compared to the ET-1 control of 2.89 ± 0.29 mmHg (n=4-8, $p<0.01$).

The combination of BQ123 and BQ788 (both 3 μM) significantly inhibited the ET-1 induced increase in PIP from 2.89 ± 0.29 mmHg to 0.89 ± 0.24 mmHg (n=4-8, $p<0.001$; see Figure 3.35) for a 400 pmol bolus dose.

With regard to the increase in LW observed with ET-1 (1.04 ± 0.35 g for a 400 pmol dose, n=8), the ET_A receptor antagonist BQ123 (3 μM) significantly attenuated the increase seen, with a 400 pmol bolus reducing the increase in LW to 0.05 ± 0.03 g (n=4, $p<0.05$). However the ET_B receptor antagonist BQ788 had no significant effect (0.45 ± 0.15 g, n=4, for a 400pmol dose). The combination of BQ123 and BQ788 (3 μM) also attenuated the ET-1 induced increase in LW (400 pmol producing an increase in LW of 0.025 ± 0.025 g, n=4, $p<0.05$; see Figure 3.35).

Endothelin-3

The addition of ET-3 in the presence of the endothelin ET_A selective receptor antagonist BQ123 (10 μM) did not alter the rise in PPP observed (400 pmol producing an increase of 5.00 ± 0.50 mmHg when compared to the ET-3 control of 4.25 ± 0.59 mmHg, n=5-6). The ET_B receptor antagonist BQ788 (3 μM) significantly lowered the ET-3 induced increases in PPP, with a 400 pmol bolus dose producing an increase in PPP of 1.33 ± 0.44 mmHg when compared to the control increase of 4.25 ± 0.59 mmHg (n=5-6, $p<0.05$).

The maximal ET-3 induced increase in PIP was not affected by the addition of 10 μM BQ123, although the curve was displaced to the left, this shift was not significant (for example, the 400 pmol bolus dose increased PIP by 5.16 ± 0.17 mmHg in the presence of 10 μM BQ123, compared to the ET-3 control value of 3.42 ± 0.35 mmHg, n=3-6). The ET_B receptor antagonist BQ788 significantly attenuated the ET-3 induced increase in PIP, with the higher doses (400 - 1600 pmol) all being significantly reduced (the 400 pmol ET-3 induced increase in PIP was reduced from 3.42 ± 0.35 mmHg to 0.50 ± 0.00 mmHg in the presence of BQ788, n=3-6, $p<0.001$, see Figure 3.36).

BQ123 (10 μM) had no effect upon the ET-3 induced increase in LW, up to 800 pmol. However, BQ788 (3 μM) did inhibit the increase in LW induced by ET-3 at higher doses (800 - 1600 pmol). For example, 800 pmol ET-3 increased LW by 0.17 ± 0.03 g in the presence of BQ788,

compared to the ET-3 control value of 2.35 ± 0.63 g (n=3-6, p<0.001, see Figures 3.36 + 3.40).

Sarafotoxin 6c

Inclusion of the ET_B receptor antagonist BQ788 (3 μM) inhibited the Sx6c induced increase in PPP, PIP and LW. 400 pmol Sx6c, in the presence of 3μM BQ788 produced an increase in PPP of 1.67 ± 0.33 mmHg, compared with the control Sx6c increase of 11.5 ± 0.96 mmHg (n=5-6, p<o0.001). PIP increased by 1.33 ± 0.17 mmHg in the presence of 3 μM BQ788, compared to the control of 8.7 ± 0.59 mmHg (n=5-6, p<0.001), with a 400 pmol bolus dose of Sx6c. The increase in LW in the presence of BQ788 was 0.08 ± 0.05 g, contrasted with the 5.32 ± 1.65 g increase in the Sx6c control (n=5-6, p<0.001; see Figures 3.37 + 3.41).

Big Endothelin-1

The ECE inhibitor phosphoramidon (1 μM) inhibited the big ET-1 induced increase in PPP, PIP and LW, with a 400 pmol bolus, in the presence of phosphoramidon, producing 2.58 ± 0.66 mmHg, 0.7 ± 0.1 mmHg and 0.03 ± 0.04 g increases in PPP,PIP and LW respectively. These contrast with the increases seen with big ET-1 of 5.58 ± 0.42 mmHg, 2.35 ± 0.31 mmHg and 0.32 ± 0.08 g, respectively (n=6-8, see Figures 3.38 + 3.42).

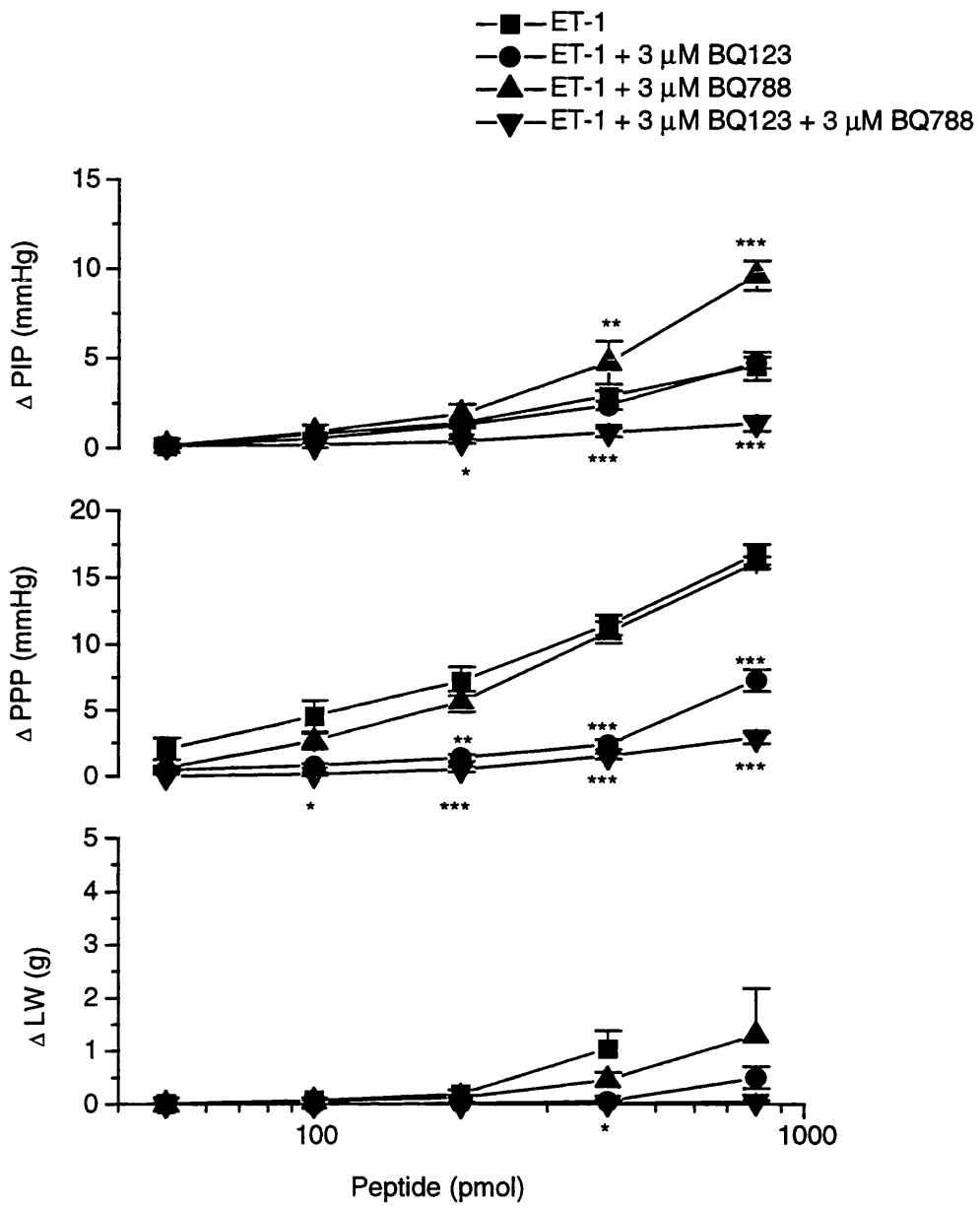

Figure 3.35. The effects of BQ123 and BQ788 both alone and in combination on bolus administration of Endothelin-1 on changes in Pulmonary Inflation Pressure (PIP), Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in isolated, ventilated perfused rat lungs. n=4-8, * $p<0.05$, ** $P<0.01$, *** $p<0.001$ vs Endothelin-1.

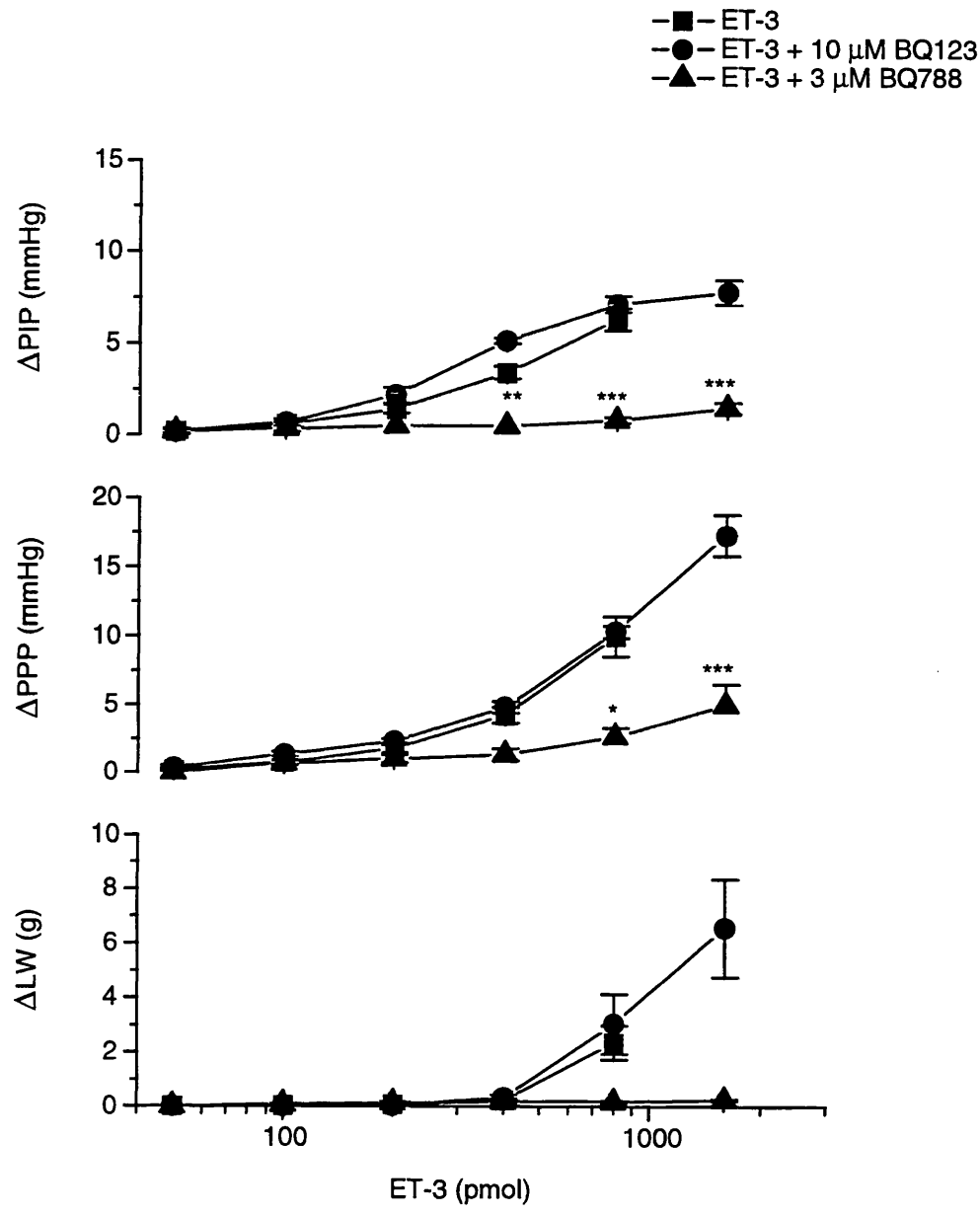

Figure 3.36. The effects of BQ123 and BQ788 on bolus administration of Endothelin-3 on changes in Pulmonary Inflation Pressure (PIP), Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in isolated, ventilated perfused rat lungs. n=3-6, * $p<0.05$, ** $P<0.01$, *** $p<0.001$ vs Endothelin-3.

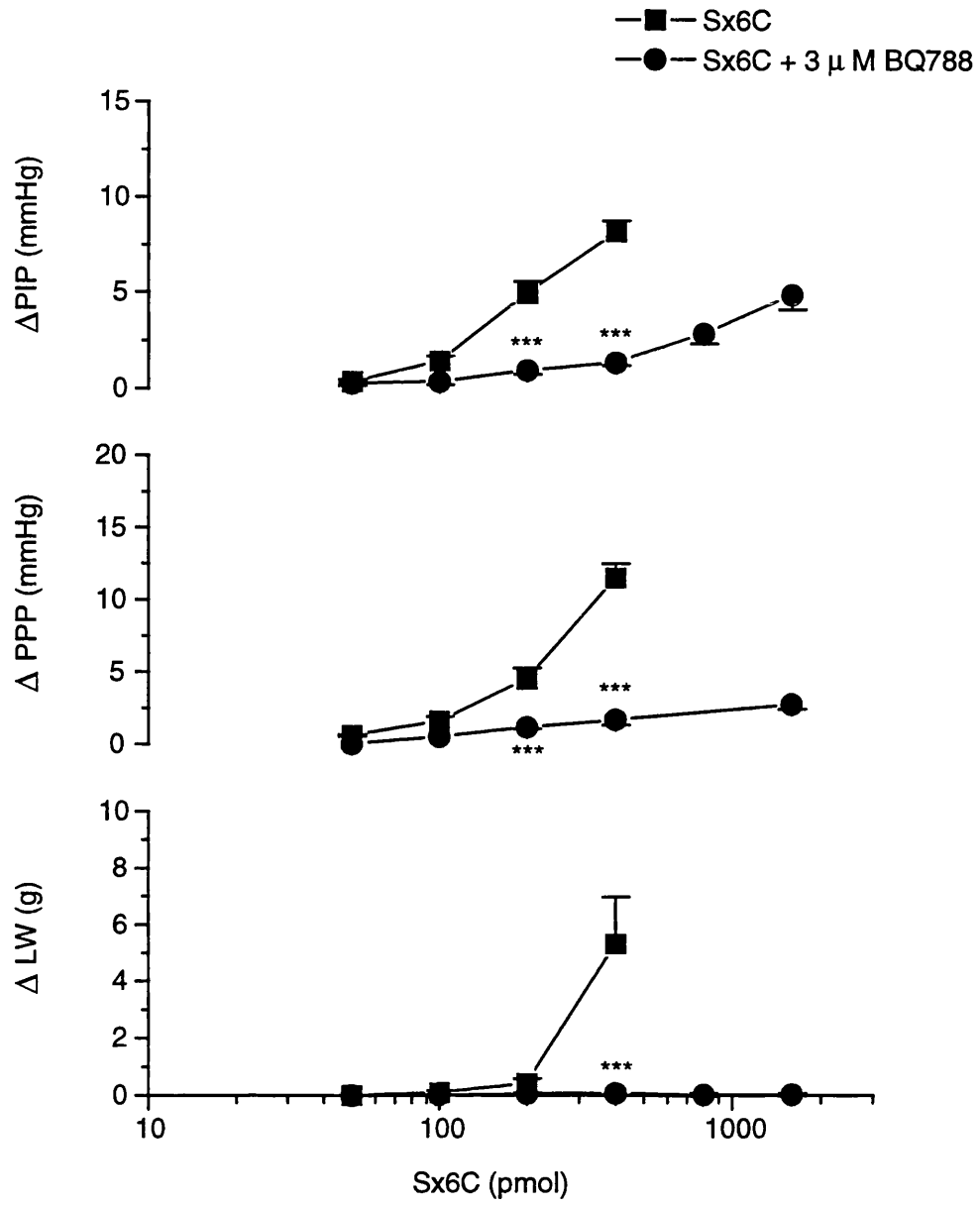

Figure 3.37. The effect of BQ788 on bolus administration of Sarafotoxin 6c on changes in Pulmonary Inflation Pressure (PIP), Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in isolated, ventilated perfused rat lungs. n=5-6, *** $p<0.001$ vs Sarafotoxin 6c.

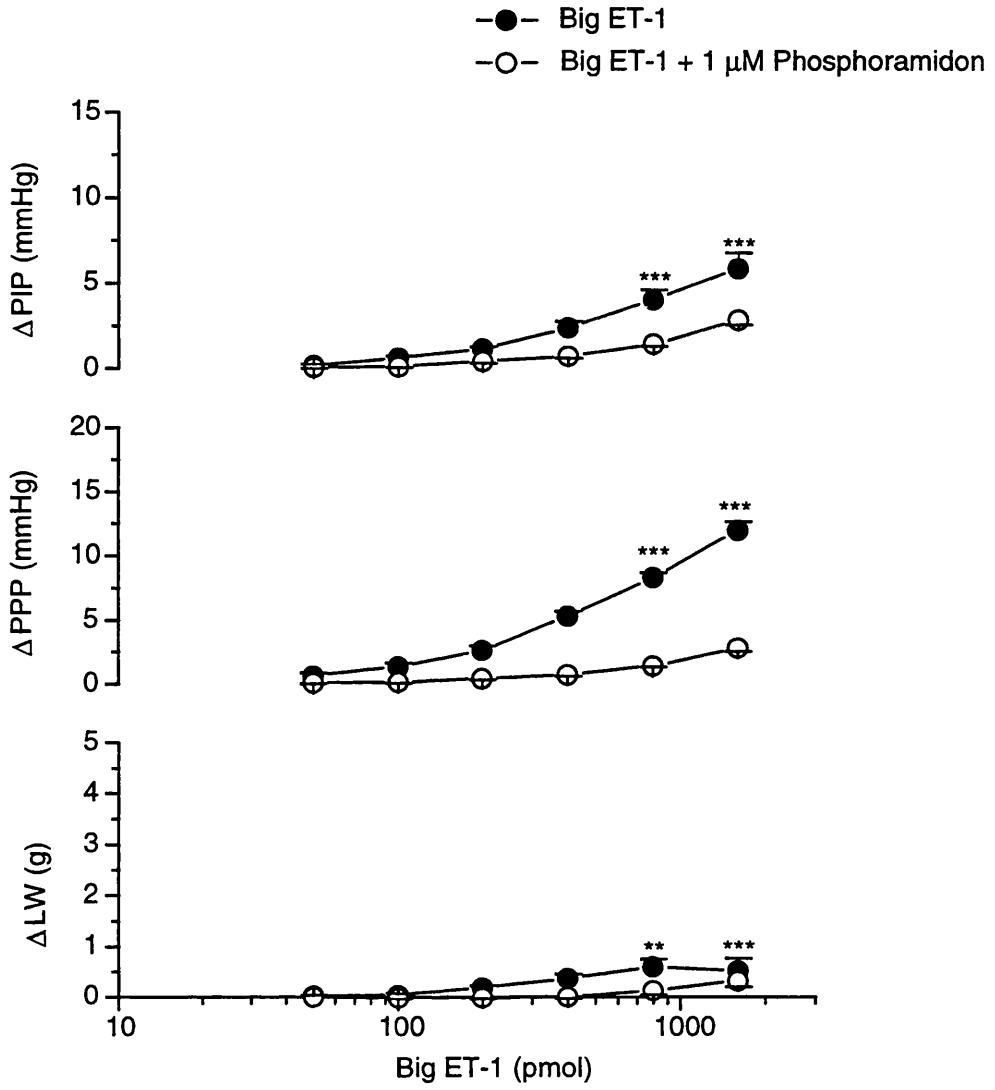

Figure 3.38. The effect of Phosphoramidon on bolus administration of Big Endothelin-1 on changes in Pulmonary Inflation Pressure (PIP), Pulmonary Perfusion Pressure (PPP) and Lung Weight (LW) in isolated, ventilated perfused rat lungs. n=6-8, ** $p<0.01$, *** $p<0.001$ vs Big-Endothelin-1.

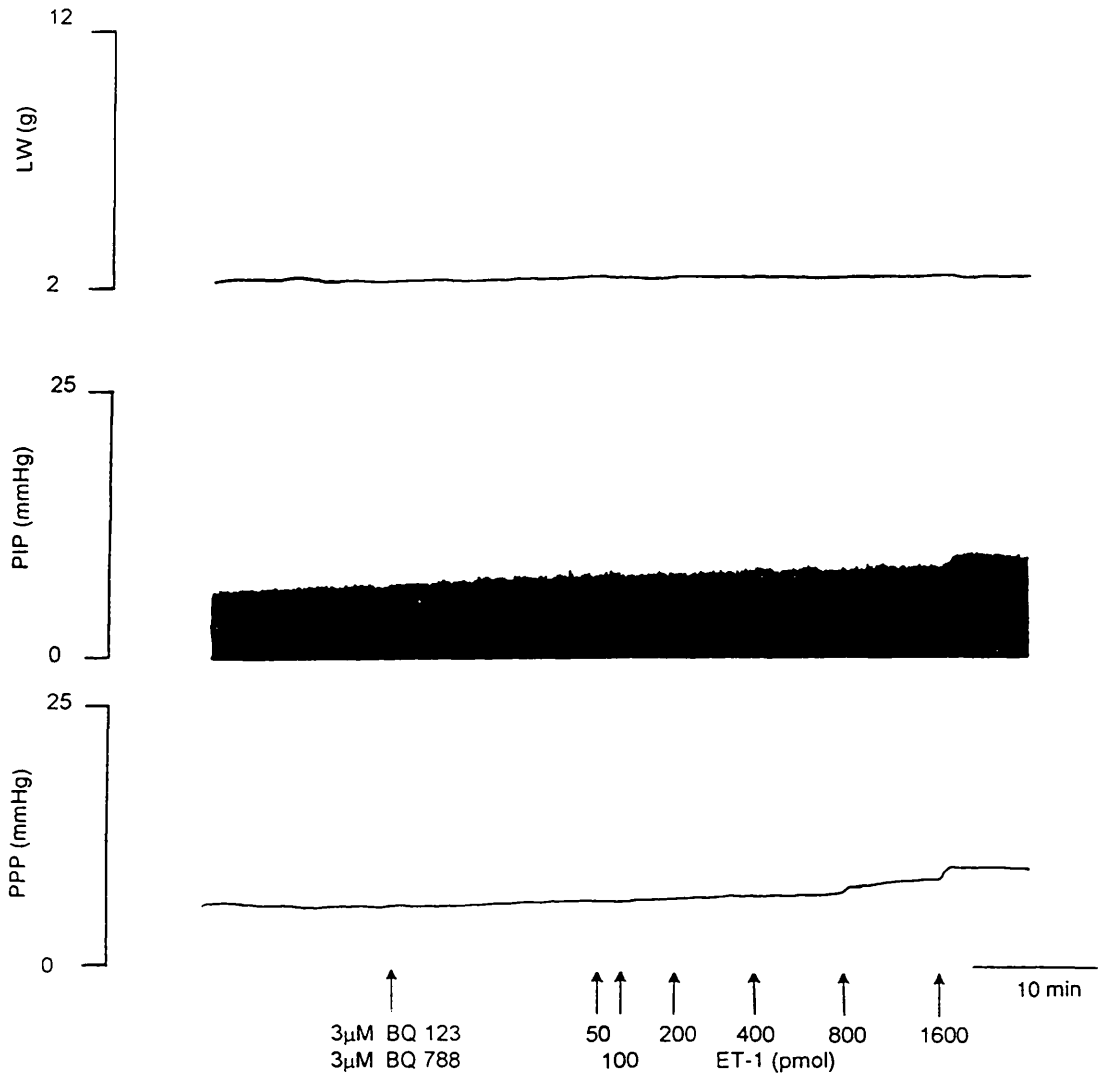

Figure 3.39. The effect of BQ 123 and BQ788 in combination on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) following bolus Endothelin-1 administration in an isolated, ventilated perfused rat lung (representative trace from 4 similar experiments).

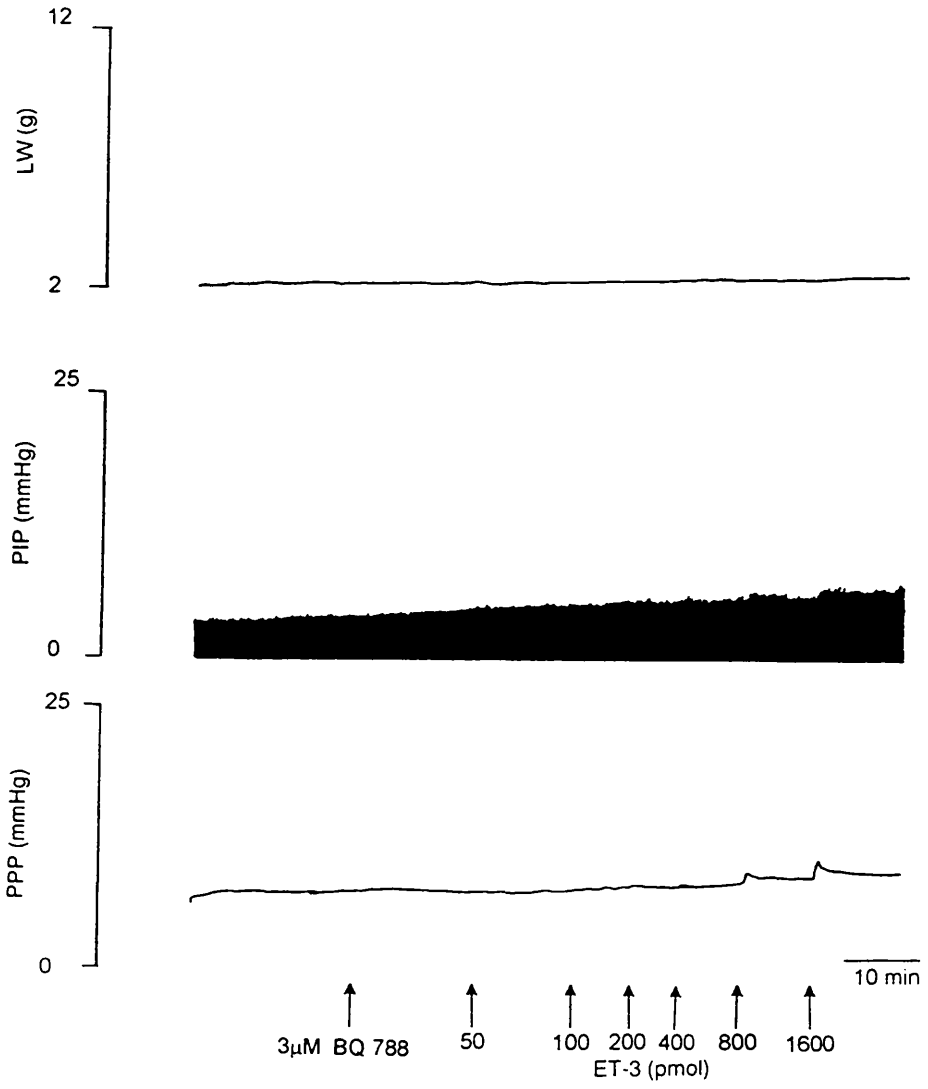

Figure 3.40. The effect of BQ788 on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) following bolus Endothelin-3 administration in an isolated, ventilated perfused rat lung (representative trace from 3 similar experiments).

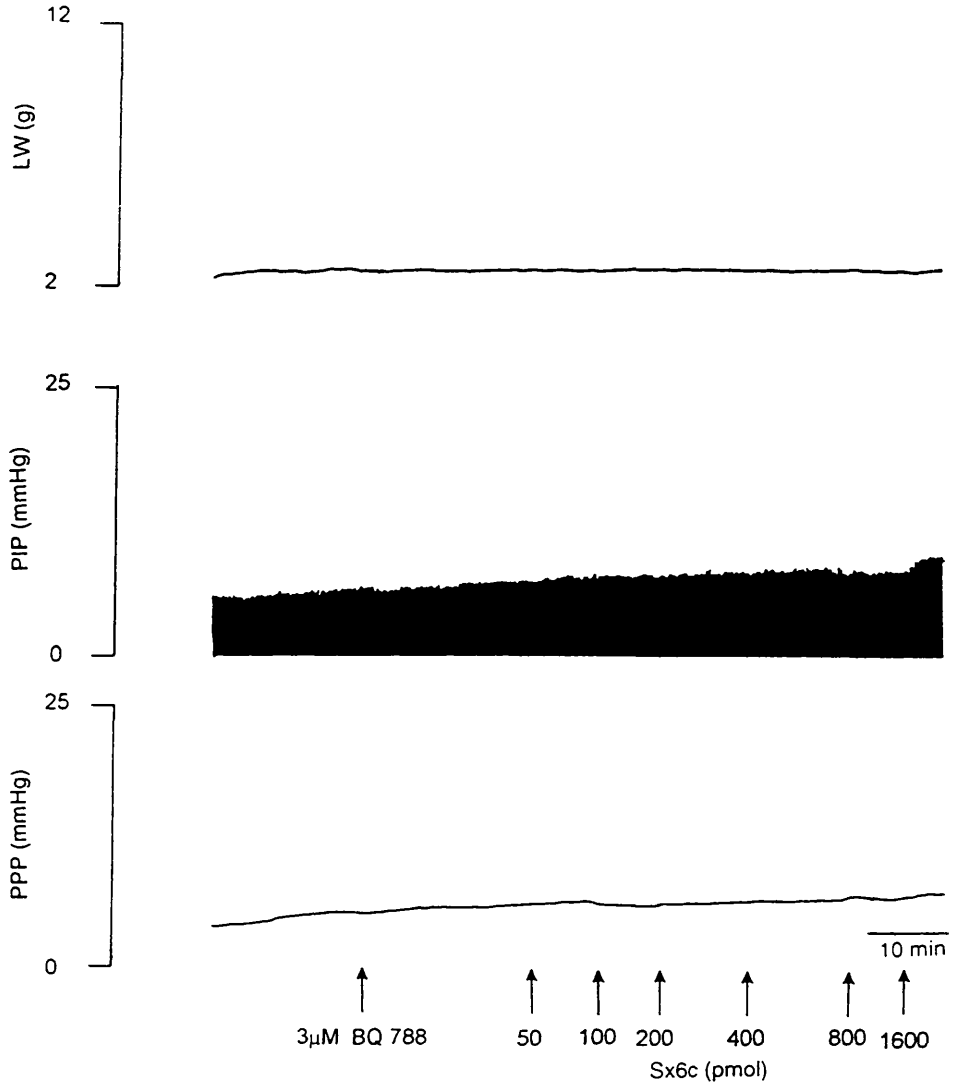

Figure 3.41. The effect of BQ788 on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) following bolus Sarafotoxin 6c administration in an isolated, ventilated perfused rat lung (representative trace from 6 similar experiments).

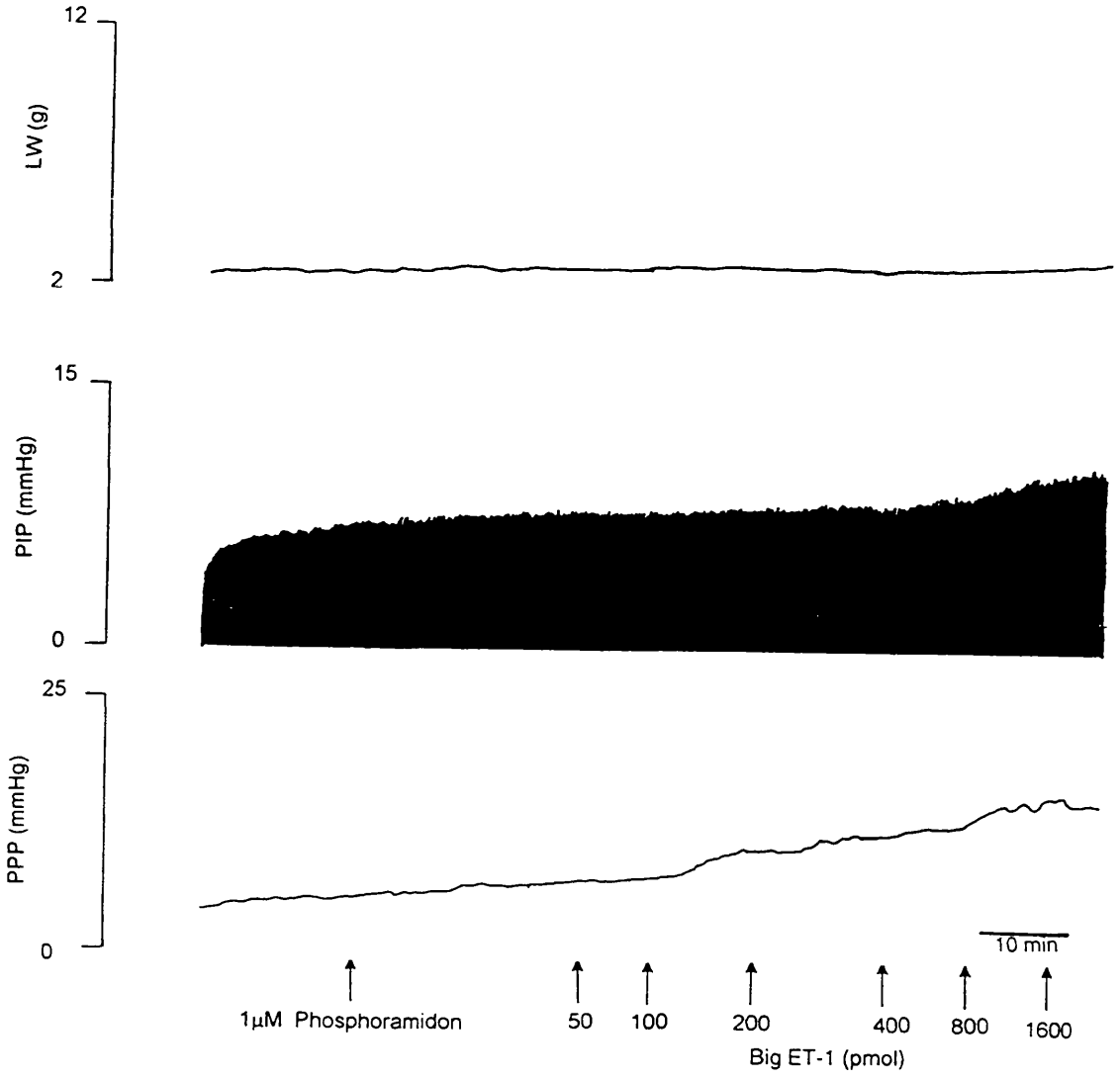

Figure 3.42. The effect of Phosphoramidon on Pulmonary Perfusion Pressure (PPP), Pulmonary Inflation Pressure (PIP) and Lung Weight (LW) following bolus Big Endothelin-1 administration in an isolated, ventilated perfused rat lung (representative trace from 6 similar experiments).

SECTION 4

Discussion

In this series of experiments, the involvement of endothelins in the responses to systemic hypoxia in an isolated perfused rat lung has been investigated. The responses to hypoxia could be inhibited by a variety of agents which have been shown to inhibit ET formation, secretion and action. In addition to this, an increase in perfusate levels of ET-1 and an increase in the lung levels of prepro ET-1 mRNA had been detected, which indicate that hypoxia stimulates ET-1 production in this model.

The use of isolated perfused lungs to study aspects of pulmonary function is well known. However the criteria for the stability of the preparation are varied. It has been demonstrated that lungs perfused under normoxic conditions in this model and in this study are stable for 2 hours, with no increase in PPP, PIP or LW and that the responses to agonists (phenylepherine, carbachol, bradykinin and angiotensin-1) are reproducible throughout the 2 hour period (Lal, 1995).

The use of a single pass perfusion model allows for removal of substances robust enough to withstand passage through the lung, which may, in reperfused lungs, continue to act on subsequent passages, complicating interpretation of the results. It should be noted that the addition of a plasma expander to the perfusate (for example dextran, ficoll or albumin), to offset oedema formation in the perfused lung, has been widely reported in the literature (Selig *et al.,* 1988; Drazen *et al.,* 1989; Westcott *et al.,* 1990; Czartolomna *et al.,* 1991;

Seale *et al.,* 1991; Pino *et al.,* 1992; Bonvallet *et al.,* 1993; Uhlig and Wollin, 1994). The omission of a plasma expander in the perfusate was a considered choice, due to the expense.

The perfusion rate used in this study (5 ml min^{-1}) has been shown to be a rate which allows maximum stability of the preparation with minimum oedema formation over the time course of the experiment. Increasing the flow rate has been shown to produce a more rapid development of oedema (Lal, 1995). In addition to this, Fisher *et al.,* (1980) demonstrated that rat lungs perfused with a Krebs' solution at a rate of 12 - 25 ml min^{-1} showed development of gross alveolar oedema after 1 hour.

The low pulmonary inflation rate and inflation volume were used to prevent stress damage to the vasculature (West and Mathieu-Costello, 1992), as even moderate over-inflation has been shown to cause lung injury (Cilley *et al.,* 1993). The normal tidal volume for a male Wistar rat of the weight used in this study is 1.6 ml, with a respiratory rate of 90 breaths min^{-1} (Flecknell, 1995). In this study, we have used a tidal volume of 1ml, which although lower than the rat tidal volume, has been shown to allow measurement of changes in airway tone in this model (Lal *et al.,* 1994). The relatively low inflation rate used was chosen to prolong the viability of the preparation.

In preliminary studies with hypoxic perfusion, it was noted that the use of standard silicone laboratory tubing with the isolated lung apparatus allowed oxygen diffusion both into and out of the equilibrated Kreb's buffer, dependent upon the gassing regimen used, thus altering the PO_2. To combat this, and prevent the PO_2 falling under normoxic conditions or increasing under hypoxic conditions, all perfusion and ventilation tubing was switched to proprietary brands (Tygon and PharMed) which report low oxygen permeability. Changing the tubing resulted in a reduction in diffusion of oxygen into the Krebs from the atmosphere, and allowed greater control and stability of perfusate PO_2, both under normoxic and hypoxic conditions.

The use of normoxic Krebs gassed with either 95% O_2 or 20% O_2 had no effect upon the viability of the preparation. For the remainder of the study, normoxic perfusion was performed with the Krebs gassed with 20% O_2 as this was deemed to be of a greater physiological relevance than 95% O_2.

EFFECTS OF HYPOXIA ON THE ISOLATED RAT LUNG

Hypoxic perfusion of the isolated ventilated lung, in a single pass manner, resulted in a slow increase in PPP, followed by an increase in LW. The increase in PPP is consistent with the phase 2 HPV reported by Jin *et al.* (1992) and Ward and Robertson (1995). The mechanisms underlying the sensing of the fall in PO_2 and the subsequent vasoconstriction seen are not yet completely understood, but data presented in this thesis indicates that endogenous endothelin could be involved in mediating the responses seen (see later).

The lack of a phase 1 response, in this study, cannot be accounted for, although it should be noted that the majority of phase 1 responses are seen in pre-constricted pulmonary vessels, or isolated lungs with raised vascular tone. Indeed, it has been reported that the magnitude of HPV seen in isolated rings is proportional to the level of pre-stimulation of the vessels with any given agonist, and the severity of the hypoxia (Rodman *et al.,* 1989). The fact that an exogenous constrictor agent was not used in this study could explain the lack of a phase 1 response.

In addition to the single pass perfusion system, a recirculating method was developed. This was used to investigate the release of ET(s) from the lung in response to hypoxia. To accomplish this, the same perfusion parameters as for single pass perfusion were used, with the

perfusate volume reduced to 50 ml, which was gassed with the appropriate humidified gas and recirculated.

Following hypoxic perfusion in the recirculating system, the PPP and LW increased, but not to the same degree as in the single pass system (see Figures 3.2 and 3.14). This discrepancy could be due to the release of endogenous vasodilator substances that have a persistent action in recirculating Kreb's perfusate (Chang and Voelkel, 1992). As with the single pass system, no significant change in PIP was observed.

The α_1-selective antagonist prazosin, at a concentration which has been shown to inhibit the phenylepherine induced increase in PPP (Lal, 1995), had no effect on the responses to single pass hypoxia in the model, indicating that activation of α_1 adrenoceptors played no role in the development of HPV. This is in agreement with the findings of Fishman (1976) and McLean (1986).

The cyclo-oxygenase inhibitor indomethacin, which at the concentration used, effectively inhibits the production of prostacyclin and thromboxane A_2 in isolated perfused rat lungs (Chang and Voelkel, 1992) inhibited HPV, but not the associated increase in LW. This is in contrast to reports indicating that inhibition of cyclo-

oxygenase enhances HPV, presumably through inhibition of endogenous vasodilator prostanoids (Haynes *et al.,* 1988; Ward and Robertson, 1995). The lack of effect of indomethacin on the increase in LW in the present study would indicate that prostanoids are not involved in mediating the LW changes, and that the increase in LW is as a result of something other than a hydrostatic oedema secondary to a vascular constriction.

It has been shown that although hypoxia reduces NO production in proximal pulmonary arteries, production is enhanced in the small arteries (Kovitz *et al.,* 1993). Another report suggested that although NO may be important in the regulation of vascular tone and may have a modulatory role in hypoxia, it is unlikely to be a major factor in the development of HPV (Ward and Robertson, 1995).

To investigate the possibility that a decrease in NO could be the cause of the increase in vascular resistance following hypoxia, NO synthesis was inhibited prior to hypoxia with the NOS inhibitors nitro-L-arginine and nitro-L-arginine methyl ester (L-NAME). These agents were used at concentrations that have been demonstrated to block both the constitutive and inducible form of NOS (Griffith and Kilbourn, 1996). The NOS inhibitors had no effect on the basal vascular resistance, and had no effect on the hypoxia-induced increase in vascular resistance, which is in agreement with previously published data (Jin *et al.,* 1992;

Leach *et al.,* 1994), and suggests that in this model a decrease in the release of the vasodilator NO is not the cause of the HPV. However these results contrast with the results of Ogata *et al.* (1992), who showed that N(G)-monomethyl-L-arginine enhanced the hypoxia induced vasoconstriction in isolated pulmonary artery rings. In addition, Sprague *et al.* (1992) have demonstrated that L-NAME produced dose dependent decreases in blood flow to the nitrogen ventilated lung in an animal model of unilateral alveolar hypoxia.

It should be noted that L-NAME prevented the increase in LW caused by hypoxia in this model. The lack of effect of nitro-L-arginine with regard to the induced increases in LW cannot be explained, as L-NAME is metabolised to nitro-L-arginine within the cell, and thus the active inhibitor is the same (Griffith and Kilbourn, 1996). This suggests that L-NAME may have some actions independent of NOS inhibition.

The NOS substrate L-arginine, in the absence of nitro-L-arginine and L-NAME, inhibited the HPV, possibly through increasing the production of the dilator substance NO. The lack of effect of L-arginine on the hypoxia-induced increase in LW provides further evidence for the independent nature of changes in vascular resistance and lung weight in this model. Together, these results show that there is no evidence for NO involvement in the development of HPV in this model.

Following the development of an isolated perfused rat lung model that responded to systemic hypoxia, and evidence in the literature that endothelins were released in response to hypoxia in several species (Perreault *et al.,* 1990; Rakugi *et al.,* 1990; Horio *et al.,* 1991; Shirakami *et al.,* 1991), the role of endothelins in the responses to hypoxia was investigated. This was accomplished by using the endothelin ET_A receptor antagonist BQ123 (Ihara *et al.,* 1992), the endothelin ET_B receptor antagonist BQ788 (Ishikawa *et al.,* 1994) and the mixed endothelin ET_A/ET_B receptor antagonist bosentan (Clozel *et al.,* 1994).

BQ123, an ET_A receptor antagonist (Ihara *et al.,* 1992), inhibited the increase in vascular resistance, and the increase in LW in a concentration dependent fashion in the single pass system. In the recirculating system BQ123 also blocked both the increase in PPP and LW, indicating a role for ET_A receptors in the responses observed. This is in agreement with results published showing that BQ123 can prevent the development of pulmonary hypertension following exposure to chronic hypoxia (Bonvallet *et al.,* 1993; Chen *et al.,* 1993; DiCarlo *et al.,* 1994; Oparil *et al.,* 1995). However it should be noted that the above studies were performed *in vivo*, with exposure to hypoxia over a long period of time. The results from the current study suggest that endothelin involvement in the responses to hypoxia can be demonstrated over a much shorter time scale (90 min). In contrast to this Takeoka *et al.* (1995) demonstrated that BQ123 had no effect

upon HPV in an isolated rat lung, however the study involved the investigation of a 5 min exposure to hypoxia, which is consistent with the phase 1 response seen in acute alveolar hypoxia. It is thought that the initial phase 1 HPV is due to depolarisation of the cell *via* closure of an oxygen-sensitive potassium channel (Osipenko *et al.,* 1995; Weir and Archer, 1995; Osipenko *et al.,* 1997), and as stated earlier, this acute response was not observed when hypoxia was induced systemically.

The endothelin ET_B receptor antagonist BQ788 (Ishikawa *et al.,* 1994) also attenuated the HPV and increase in LW, both in the single pass and recirculating systems. This is in contrast to the findings of Okada *et al.* (1995), who demonstrated that ET_B receptor blockade with RES-701-1 increased pulmonary artery pressure in pulmonary hypertension. However, recent studies by Lal *et al.* (1996) and Muramatsu *et al.* (1997) have demonstrated the presence of both ET_A and ET_B receptors in the lung, and that these receptors contribute to the ET induced vasoconstriction. BQ788 has also been shown to inhibit ET-1 induced vasoconstriction in human isolated pulmonary vessels (McCulloch *et al.,* 1996). It is possible that the ET_{B2} receptor subtype, which has been demonstrated to mediate vasoconstriction, is involved in the responses seen to hypoxia in this model.

The mixed ET_A/ET_B receptor antagonist bosentan (Clozel *et al.,* 1994) blocked the increases in LW, caused by hypoxia, in a concentration dependent manner, however the blockade of HPV only occurred at the lower concentration (1.5 μM). In the recirculating system, bosentan, at the concentration used (1.5 μM), also inhibited the hypoxia-induced increases in PPP and LW. This blockade of HPV is in agreement with the work of Chen *et al.* (1994), Chen *et al.* (1995), Eddahibi *et al.* (1995) and Willett *et al.* (1997) who have shown that mixed ET_A/ET_B receptor antagonists can prevent and reverse the development of pulmonary hypertension in several models, both *in vitro* and *in vivo.*

The lack of a concentration-related effect of bosentan on blocking the increase in vascular resistance cannot be explained, although Xia and Nye (1995) have shown that the related compound Ro46-2005, which has a similar structure to bosentan, displays non concentration-dependent effects in rat resistance pulmonary artery rings. It may be that bosentan is acting as a partial-agonist in the isolated lung.

Therefore, this data presents evidence that ET(s) are involved in the responses to hypoxia in this model. To investigate if the ET(s) are derived from increased synthesis or release from storage vesicles, an ECE inhibitor and agents that interfere with release and synthesis were utilised.

The role of big endothelin(s) was investigated by using the endothelin converting enzyme inhibitor phosphoramidon (Fukuroda *et al.,* 1990; Matsumura *et al.,* 1990; McMahon *et al.,* 1991; Sawamura *et al.,* 1991). The results indicated that the production of endothelin from big endothelin is involved in the responses seen, as the converting enzyme inhibitor, phosphoramidon, attenuated both the increase in vascular resistance and the increase in lung weight following exposure to hypoxia, both in the single pass and recirculating systems. This is in agreement with the work of Stadnicka *et al.* (1995) who showed that phosphoramidon could reduce the extent of hypoxic constriction in rabbit mesenteric vessels. Vemulapalli *et al.* (1992) have demonstrated that phosphoramidon can abolish the increase in ET-1 release following ischaemia in the isolated perfused guinea-pig lung. However it was later shown that phosphoramidon did not inhibit endogenous ET-1 release following hypoxia in the rat, when plasma levels were measured (Vemulapalli *et al.,* 1994), however the source of the ET-1 could not be identified. The location of the conversion of the big ET(s) to the mature peptide(s) is not known, however it has been demonstrated that phosphoramidon can enter cells, and it will prevent both the intracellular and the extracellular conversion of the big ET(s) (Sawamura *et al.,* 1991). The subtype of the ECE involved in the conversion of big ET to the mature peptide cannot be identified, as phosphoramidon has been shown to inhibit both the ECE-1 and the ECE-2 isoforms (Xue *et al.,* 1994; Emoto and Yanagisawa, 1995).

It is interesting to note the lack of an increase in PIP following exposure to hypoxia. If ETs are involved in the responses observed following the onset of hypoxia, then it would be expected that PIP would increase, as endothelins have been shown to increase airway resistance following bolus administration *via* the pulmonary artery (Lal *et al.,* 1995). However, the effect of hypoxia on airway resistance has not been reported in the literature. The lack of an increase in PIP may be due to the type of the hypoxia (systemic as opposed to alveolar), and if ET(s) are being released from the endothelium, the release would probably be polar in nature, and directed towards the vascular smooth muscle cells (Yoshimoto *et al.,* 1991; Wagner *et al.,* 1992).

Following the observation that the ET_A receptor antagonist BQ123, at the lower concentration used (3 μM), inhibited the hypoxia-induced increase in PPP, but had no significant effect upon the hypoxia-induced increase in LW, and the higher concentration of BQ123 used (10 μM) inhibited both the increase in PPP and the increase in LW following hypoxia, the possibility of different mechanisms for increases in both PPP and LW was raised. To investigate this possibility further, the F-actin stabilising agent phalloidin (Estes *et al.,* 1981) was used, as it had been demonstrated by Kuroes *et al.* (1993) that phalloidin could inhibit the ET-3 induced increase in vascular permeability in superfused rat mesentery, without inhibiting the observed ET-3 induced vasoconstriction. However, it was found that phalloidin

attenuated both the increase in PPP and LW following exposure to hypoxia both under single pass and recirculating conditions. It has been reported by Tasaka and Kitazumi (1994) that F-actin plays a role in the secretion of ET-1 from endothelial cells. As a result, the phalloidin could well be preventing the release of ET(s) from the endothelium, and therefore the inhibition of the hypoxia-induced increase in PPP and LW observed following hypoxia may due to the inhibition of ET secretion within the lung.

To investigate the involvement of the cytoskeleton in the responses to hypoxia further, colchicine, an agent which disrupts microtubules (Borisy and Taylor, 1967), which have been reported to be involved in the secretion of ET-1 (Kitazumi *et al.,* 1991), was utilised, again under both the single pass and recirculating protocols. The increases in PPP and LW were inhibited by colchicine following hypoxic exposure, indicating that ET release from the cells within the lung involves the microtubules.

Initial reports indicated that there were no storage sites within cells for ET-1 (Nakamura, *et al.,*1990), although endothelial cells may posses some preformed ET-1 that stimuli, such as stretch, can release immediately (Harrison *et al.,* 1993; Macarthur *et al.,* 1994; Harrison *et al.,* 1995). To investigate the nature of the source of the ET involved in responses to hypoxia in this model (i.e. release from pre-formed

stores or *de novo* synthesis), the peptide synthesis inhibitor cycloheximide was used (Obrig *et al.,* 1971). If the responses to hypoxia are as a result of release of pre-formed ET(s), then pre-treatment of the lung with cycloheximide would have no effect. If, however, the ET release was as a result of *de novo* synthesis, then pre-treatment with cycloheximide would be expected to prevent the hypoxia-induced increase in LW and PPP. This is indeed was what was observed when cycloheximide was given prior to hypoxia, both under the single pass and recirculating systems, indicating that ET release is as a result of *de novo* synthesis.

To investigate the mechanisms involved in the hypoxia-induced increase in LW, an albumin bound Evans Blue dye infusion was used to investigate changes in vascular permeability. Increased albumin bound Evans Blue dye accumulation within the tissue is indicative of a permeability change within the lung vasculature (Lal *et al.,* 1995). In the present study, it has been shown that the increases in LW produced by exposure to hypoxia are associated with a large accumulation of albumin bound dye. This suggests that the increase in LW following exposure to hypoxia is as a result of a permeability change within the pulmonary vasculature and not a simple hydrostatic oedema.

PERFUSATE ENDOTHELIN-1 LEVELS

Effective concentrations of agents shown to attenuate both HPV and the associated increase in LW were also used in the recirculating system, and the perfusate samples collected for ET extraction. Extraction efficiency of the Amprep ethyl C2 columns has been demonstrated to be 75% (A. White, personnel communication).

The levels of ET-1 in the tissues exposed to hypoxia had increased by approximately four fold when compared to time-matched normoxic controls. Increased ET-1 secretion, in response to hypoxia, has also been seen in human umbilical vein endothelial cells (HUVECS; Kourembanas *et al.,* 1991; Gertler and Ocasio, 1993; Kourembanas, *et al.,* 1993), bovine coronary artery endothelial cells (Hieda and Gomez-Sanchez, 1990), human proximal tubular cells (Ong *et al.,* 1995) and cardiomyocytes (Kagamu *et al.,* 1992). It has been shown that in pulmonary artery endothelial cells hypoxia can increase (Golden *et al.,* 1995), have no effect on (Hassoun *et al.,* 1995) or decrease (Wiebke *et al.,* 1992; Markewitz *et al.,* 1995) ET-1 secretion. Plasma ET-1 levels are also increased in animals and humans exposed to alveolar hypoxia for more than one hour (Horio *et al.,* 1991; Shirakami *et al.,* 1991; Elton *et al.,* 1992; Perella *et al.,* 1992; Li *et al.,* 1994a; DiCarrlo *et al.,* 1995; Goerre *et al.,* 1995; Oparil *et al.,* 1995).

It has been demonstrated that in animals exposed to alveolar hypoxia and humans exposed to high altitude the increase in plasma ET-1 correlates with the decrease in PO_2 (Horio *et al.,* 1991; Shirakami *et al.,* 1991; Goerre *et al.,* 1995).

It should be noted that back calculation of the level of ET-1 measured in the perfusate falls within the range administered as bolus doses in experiments in this study. Assuming a polar secretion for ET-1 from endothelial cells, the amount of ET-1 in the perfusate equates to a circulating concentration of approximately 5 nM following 90 min hypoxia, which equates to a bolus administration of 200 - 400 pmol, indicating that the amount of ET-1 released is in the range to cause the vasoconstriction and increase in lung weight observed following hypoxic exposure.

The endothelin receptor antagonist BQ123 lowered perfusate levels of ET-1 following the onset of hypoxia. This is a surprising result, as the ET-1 level would be expected to increase following ET_A receptor blockade due to ET-1 displacement from the receptors. The reported IC_{50}, obtained from ligand binding assays, using radiolabelled ligand, for ET-1 and BQ123 on cloned human ET_A receptors is reported to be 0.21 and 13 nM respectively (Dr. T. Brown, Rhone-Poulenc Rorer, personal communication). However, it has been reported that hypoxia increases both ET_A and ET_B receptor mRNA levels in the lung, and

this may lead to an increase in expression of the receptors (Li *et al.,* 1994b), however this was measured following chronic hypoxic exposure over a period of 4 weeks. Thus, treatment with the ET_A receptor antagonist BQ123 (Ihara *et al.,* 1992) may prevent ET-1 binding to the ET_A receptor and increase clearance *via* the ET_B receptor (Fukuroda *et al.,* 1995; Sato *et al.,* 1995), leading to a reduction in the amount of ET-1 seen in the perfusate. The reported IC_{50} value for ET-1 on a cloned human ET_B receptor is 0.1 nM (Dr. T. Brown, personal communication), indicating that the ET_B receptor has a greater affinity for ET-1 over the ET_A receptor. Support for this comes from the experiments using the ET_B receptor antagonist BQ788.

The ET_B receptor antagonist BQ788 (Ishikawa *et al.,* 1994) increased the perfusate ET-1 levels following hypoxia. This is in agreement with the findings of Fukuroda *et al.* (1994) and Sato *et al.* (1995) who showed that the ET_B receptor subtype was responsible for the removal of ET-1 from the circulation within the rat lung. Blockade of the ET_B receptor would prevent removal of the circulating ET-1, and increase the perfusate level.

However, the mixed ET_A/ET_B receptor antagonist bosentan (Clozel *et al.,* 1994) lowered perfusate ET-1 levels. This contrasts with the

results seen with BQ123 and BQ788, where ET_A receptor blockade resulted in a decrease in the perfusate level of ET-1, and ET_B receptor blockade resulted in an increase in the perfusate level of ET-1. It would be expected that combined ET_A/ET_B receptor blockade would increase ET-1 levels in the recirculating perfusate, due to blocking the ET_B mediated clearance and displacing bound ET-1 from the ET_A receptors. This theory is in agreement with the results reported by Hemsen *et al.* (1996), who showed that bosentan blocked the clearance of ET-1 from the circulation in the pig. Furthermore, Kiowski *et al.* (1995) demonstrated that bosentan doubled the plasma concentration of ET-1 in humans. The reason for this discrepancy is not clear, however it may be that bosentan may be interfering with the ECE, and altering the conversion of big ET to the mature peptide.

The decrease in perfusate ET-1 levels in the presence of the endothelin converting enzyme inhibitor phosphoramidon (Fukuroda *et al.,* 1990; Matsumura *et al.,* 1990; McMahon *et al.,* 1991; Sawamura *et al.,* 1991) is probably due to the inhibition of the conversion of big ET-1 into the mature peptide ET-1. Evidently phosphoramidon suppresses the secretion of ET-1 from endothelial cells *via* its direct inhibitory effect on endothelin converting enzyme (Ikegawa *et al.,* 1990; Budzik *et al.,* 1991; Sawamura *et al.,* 1991; Sheilds *et al.,* 1991). It has also been reported that the C-terminal fragment of big ET-1 is

also decreased by phosphoramidon, while an increased secretion of big ET-1 takes place (Tasaka and Kitazumi, 1994).

Secretion of ET-1 from cells has been reported to involve various components of the cytoskeleton (see Figure 4.1; Tasaka and Kitazumi, 1994). It has been reported that thrombin, a secretagogue of ET-1, increases polymerised tubulin content of cells without affecting the total tubulin content (Kitazumi *et al.,* 1991). From these findings, it is suggested that the microtubular system may play an important role in the secretion of ET-1. This is in agreement with the results seen in this study, where the microtubule disrupting agent colchicine (Borisy and Taylor, 1967) lowered the ET-1 release in response to hypoxia. In addition to this, the involvement of the actin/myosin system has been reported (see Figure 4.1; Kitazumi and Tasaka, 1992). This can be examined by using the F-actin stabilising agent, phalloidin (Estes *et al.,* 1981). The F-actin stabilising agent phalloidin inhibited the ET-1 secretion in response to hypoxia in this model, in agreement with the findings of Kitazumi and Tasaka (1992).

Together, these results indicate that ET-1 secretion from the lung plays an important role in the responses to hypoxia in the lung.

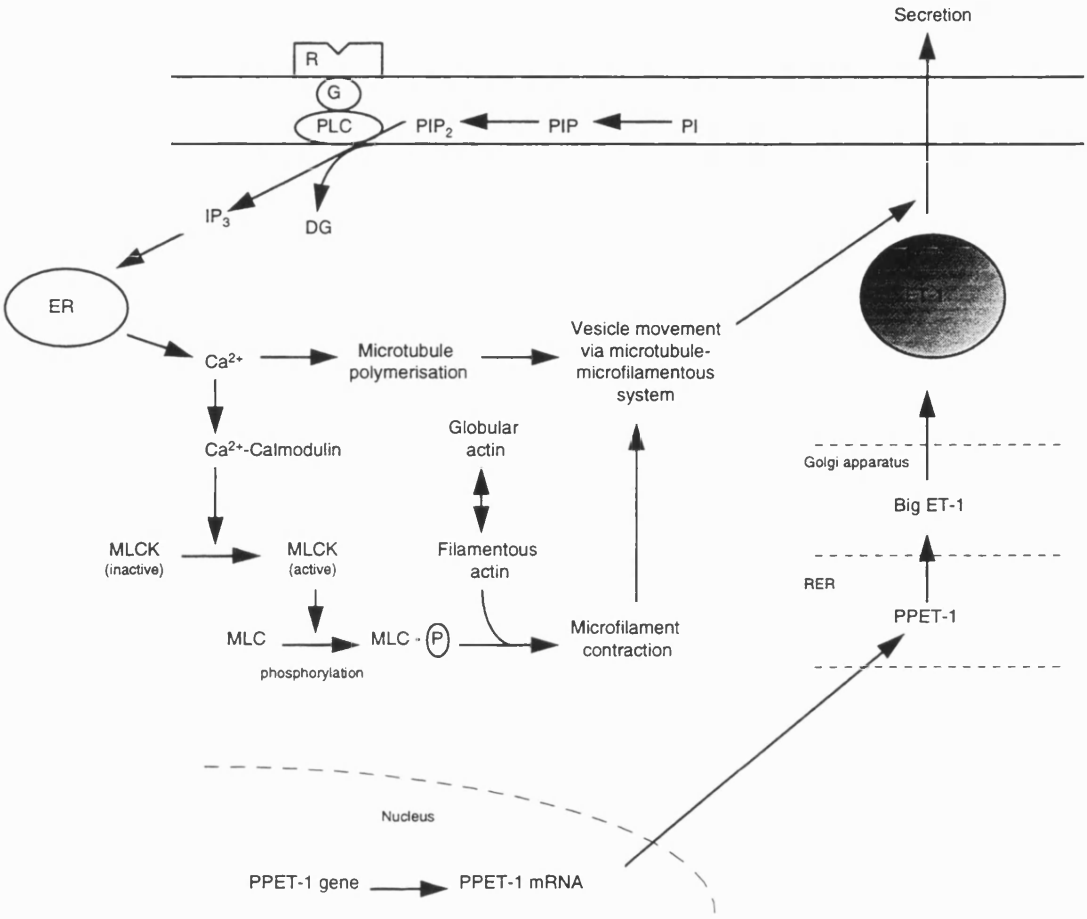

Figure 4.1. Process leading to secretion of ET-1 from endothelial cells. R, receptor; G, G-protein; PLC; phospholipase C; PI, phosphotidylinositol; PIP, phosphotidylinositol 4-monophosphate; PIP_2, phosphotidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; DG, *sn*-1,2-diacylglycerol; ER, endoplasmic reticulum; RER, Rough endoplasmic reticulum; MLCK, myosin light chain kinase; MLC, myosin light chain (reproduced from Tasaka and Kitazumi, 1994).

If ET-1 is produced as a result of *de novo* synthesis in response to hypoxia, then inhibition of peptide synthesis would result in a reduction of ET-1 production. This hypothesis was tested by utilising the peptide synthesis inhibitor cycloheximide (Obrig *et al.,* 1971) which inhibited the hypoxia-induced increase in perfusate ET-1 levels. This is in agreement with the results of Bodi *et al.* (1995) who reported that ET-1 release stimulated by hypoxia from early passage endothelial cells could be inhibited by cycloheximide.

TISSUE ENDOTHELIN-1 LEVELS

After recirculating hypoxic perfusion, lungs were snap frozen, and ETs extracted. When compared with tissues exposed to normoxic perfusion, hypoxia had no significant effect upon the tissue levels of ET-1. Similar findings have been reported by Ono *et al.,* (1992) and Stelzner *et al.* (1992) who found no increase in whole lung ET-1 levels in response to hypoxia in the rat. This contrasts with the reports of Shirakami *et al.* (1991) and Li *et al.* (1994a) who observed an increase in the whole lung ET-1, following 48 hour exposure to 10% O_2. The lack of an increase in whole lung ET-1 levels can be explained, if ET-1 is produced and secreted in response to stimuli; then lung homogenate levels would expect to remain constant (providing translocation of ET-1 out of the cell is not rate limiting), as there is no evidence for storage vesicles within the cells, and synthesised ET-1

would be secreted following production. In addition to this, whole lung homogenates would contain not only cellular ET-1, but also ET-1 bound to receptors, which may be liberated by the extraction procedure and hence interfere with ET-1 measurement. It has been reported that the ET_B receptor is responsible for clearing ET-1 from the circulation in the rat (Fukuroda *e al.,* 1994; Sato *et al.,* 1995). In addition to this, following ET binding to the ET_A receptor, the whole receptor/ligand complex is internalised (Chun *et al.,* 1995). The extraction procedure used may liberate mature ET-1 from big ET-1, complicating the measurement of cellular ET-1 even further.

The lowering of the tissue endothelin levels with the ET_A receptor antagonist BQ123, as seen in this study, could be due to inhibition of ET-1 binding to the ET_A receptor subtype. If binding of ET to the receptor is impaired by the receptor antagonist, then total ET-1 in, or attached to, a cell would be reduced, and hence the total lung ET would be reduced, as more ET would be washed out of the lung in the perfusate. However this contrasts with the results reported in this study where BQ123 lowers perfusate ET-1 levels. The reason for this is not clear.

The reduction in tissue levels of ET-1 following bosentan treatment may be due to the occupancy of ET_A and ET_B receptors by the

antagonist, displacing bound ET into the perfusate and increasing removal from the tissue (Clozel *et al.,* 1994) Therefore, receptor blockade may reduce cellular levels of ET-1, which would be seen as a reduction in whole tissue levels. However in this study, bosentan lowers the perfusate ET-1 levels.

The disruption of the cytoskeletal components F-actin and tubulin by phalloidin (Estes *et al.,* 1981) and colchicine (Borisy and Taylor, 1967) would be expected to increase tissue endothelin-1 levels, as inhibition of secretion of the peptide would lead to increased accumulation within the tissue. However, if endothelins are not being secreted and consequently not removed from the circulation, then the tissue levels may not change, as any ET secreted is probably going to be taken up into the endothelium *via* internalisation after combination with its receptor. This may explain the lack of any significant effect of these agents upon the tissue levels of ET-1.

If ET-1 is produced as a result of *de novo* synthesis following hypoxia, as has been suggested by Tasaka and Kitazumi (1994), then inhibition of peptide synthesis by cycloheximide (Obrig *et al.,* 1971) should lead to a reduction of whole tissue ET-1 levels. The results obtained showed a reduction in tissue ET-1 levels, in agreement with the results of Bodi *et al.* (1995).

Increased prepro endothelin-1 mRNA levels following exposure to hypoxia have been found in the lungs of animals in a number of different studies, including those by Shirakakmi *et al.* (1991), Elton *et al.* (1992), Donahue *et al.* (1994), Li *et al.* (1994a), Li *et al.* (1994b), Oparil *et al.* (1995) and Yang *et al.* (1997). The increase in prepro endothelin-1 mRNA in the lungs is a relatively selective response to alveolar hypoxia, as it has been shown that the only other organ it occurs, in animals exposed to chronic hypoxia, is the heart (Elton *et al.,* 1992; Li *et al.,* 1994a). However, in marked contrast, Ono *et al.* (1992) and Stelzner *et al.* (1992) have reported that in rats exposed to hypoxia for three weeks there was no increase in prepro endothelin-1 mRNA levels in these animals. It has recently been reported that long term exposure to hypoxia leads to a reduction in the initial increase in mRNA levels, and this may account for the observation that prepro ET-1 mRNA levels showed no increase following three weeks exposure to hypoxia (Dr. J.C. Wanstall, personnel communication).

The results from the current study indicate that the increase in perfusate ET-1 could well be due to increased peptide synthesis following up-regulation of the endothelin gene in the lung.

Taken together, the results from the single pass studies, recirculating studies; and both the perfusate and tissue ET-1 levels and the prepro

endothelin-1 mRNA levels indicate that endothelins play an important role in the responses to hypoxia seen in this model.

ENDOTHELINS IN THE LUNG

Following the demonstration of ET-1 involvement in the responses to hypoxia in this model, the receptor subtypes involved in the responses to ETs were investigated. ET-1, ET-3 and Sx6c produced dose-dependent increases in PPP, PIP and LW following bolus administration. These findings are in agreement with the responses reported in other rat lung preparations (Rodman *et al.,* 1992; Bonvallet *et al.,* 1993; Hisaki, *et al.,* 1994a; Hisaki *et al.,* 1994b; Lal *et al.,* 1995) and the responses seen in isolated pulmonary vessels in the rat (Wanstall and O'Donnell, 1990; Rodman *et al.,* 1992; Bonvallet *et al.,* 1993; Hisaki *et al.,* 1994a; Hisaki *et al.,* 1994b).

The finding in this study that, at low doses, ET-1 is more potent than ET-3 at causing vasoconstriction indicates that this response is partly mediated by an ET_A receptor subtype (Webb, 1991). This is supported by the observation that BQ123, an ET_A specific receptor antagonist (Ihara *et al.,* 1992), caused an inhibition of the ET-1 constrictor response (Bonvallet *et al.,* 1993; Sogabe *et al.,* 1993). However the involvement of ET_B mediated constriction cannot be ruled out, as BQ123 did not cause a complete inhibition of the ET-1 induced vasoconstriction, this was achieved by using a combination of BQ123 and the specific ET_B receptor antagonist BQ788 (Ishikawa *et al.,* 1994).

BQ123 did not inhibit the ET-3 induced increase in vascular resistance, again providing evidence that the response is mediated by a combination of ET_A and ET_B receptor subtypes. The ET-3 induced increase in vascular resistance was inhibited by the ET_B selective antagonist BQ788, indicating that ET-3 is causing vasoconstriction as a result of ET_B activation.

The ET_B selective agonist Sx6c (Kloog and Sokolovsky, 1989) also caused vasoconstriction in the lung. Similar findings have been reported in the guinea-pig lung (Noguchi *et al.,* 1993) and in isolated rat pulmonary artery rings (MacLean *et al.,* 1994), in addition to the isolated rat lung (Lal, 1995).

The precursor peptide of ET-1, big ET-1, also caused vasoconstriction in this model, although with reduced potency (approximately four fold less potent than ET-1, see Figure 3.34), when compared to ET-1. It has been shown that the biological activity of big ET-1 is due to its conversion to ET-1 (McMahon *et al.,* 1991; Lehoux *et al.,* 1992; Hisaki *et al.,* 1994b). In this model, the endothelin converting enzyme inhibitor phosphoramidon inhibited the vasoconstriction caused by big ET-1, indicating the presence of a phosphoramidon sensitive ECE within the lung.

The bronchoconstrictor potency profile of the ETs (ET-1=ET-3) and the results from Sx6c experiments suggest that the ET_B receptor is

mediating the bronchoconstrictor response seen in this model. Hay *et al.* (1993) have shown that ETs induce contractions *via* the ET_B receptor in guinea-pig lung parenchymal strips. This response is probably mediated by the ET_{B2} receptors located on the bronchial smooth muscle cells. In addition to this, the ET_A receptor antagonist FR139317 has no effect on ET-1 induced contraction of guinea-pig trachea (Cardell *et al.,* 1993), which provides further evidence that bronchoconstriction is mediated by ET_B receptors.

It is interesting to note that the ET_B receptor antagonist BQ788 increased the bronchoconstrictor response to ET-1 in the airways. This is in contrast to the majority of results reported, where BQ788 inhibits ET-1 induced bronchoconstriction (Battistini *et al.,* 1994; Adner *et al.,* 1996; Nagase *et al.,* 1997; Takahashi *et al.,* 1997). This may be due to the increased amount of ET-1 available for binding to constrictor ET_A receptors, following inhibition of the ET_B receptors by BQ788. It has previously been shown that ET_A receptors are present within the lung (Henry, 1993). Alternatively, BQ788 may block a receptor-mediated release of a dilator substance, for example NO (Lal *et al.,* 1996).

The increase in airway resistance following ET-3 administration was not affected by BQ123 (Ihara *et al.,* 1992), but completely inhibited by the ET_B receptor antagonist BQ788 (Ishikawa *et al.,* 1994), indicating that the response is mediated by ET_B receptors. This is in agreement

with the observation that the Sx6c induced bronchoconstriction is inhibited by the ET_B receptor antagonist BQ788 (Ishikawa *et al.,* 1994).

The bronchoconstriction induced by big ET-1 was equal in magnitude to that observed with ET-1, which is in contrast to the responses seen in the vasculature, where ET-1 was more potent that big ET-1. This is in agreement with the findings of Held *et al.* (1997), who reported that the big ET-1 induced bronchoconstriction was more prominent than the vasoconstriction in the isolated perfused rat lung. The reasons for the increased activity of big ET-1 compared with ET-1 in the bronchial circulation are not clear, although it may be due to the presence of an ECE isoform with a greater affinity for big ET-1, or a higher level of the enzyme in the bronchial circulation. Phosphoramidon, an endothelin converting enzyme inhibitor (Fukuroda *et al.,* 1990; Matsumura *et al.,* 1990; McMahon *et al.,* 1991; Sawamura *et al.,* 1991) also inhibited the big ET-1 induced bronchoconstriction, although at much lower concentrations than reported by Held *et al.* (1997).

Results from the current study indicate that ETs are potent compounds in producing increases in lung weight, which supports the findings of Pons *et al.* (1991); Rodman *et al.* (1992) and Ercan *et al.,* (1993). The results from this study and those of Lal (1995) suggests that the changes in LW may be due to a direct effect of endothelins on vascular permeability, and not just as a result of a hydrostatic oedema following venoconstriction (Lal *et al.* 1996), which is a known result of

ET action (Horgan *et al.,* 1991; Rodman *et al.,* 1992). It may be that endothelin-induced mediator release is responsible for the increase in lung weight, as the thromboxane A_2 synthesis inhibitor UK 38485 has been shown to attenuate ET-1 induced increases in lung weight in an isolated perfused rat lung (Ercan *et al.,* 1993). However this is in contrast to the results seen in this study, where indomethacin, which would also decrease thromboxane production, had no effect upon the hypoxia induced increase in LW.

The ET_A and ET_B receptor antagonists BQ123 (Ihara *et al.,* 1992) and BQ788 (Ishikawa *et al.,* 1994) both attenuated the ET-1 induced increase in LW, however, both agents in combination were needed to abolish the response in this study. This indicates that both ET_A and ET_B receptors are involved in the endothelin-induced increase in LW, seen in both single pass and recirculating hypoxic perfusion in this model.

The ET-3-induced increases in LW were not altered in the presence of BQ123, but completely abolished by the ET_B receptor antagonist BQ788. This indicates that with regard to ET-3-induced increases in LW, this is probably mediated by the ET_B receptor, although the majority of ET_B receptors in the pulmonary vasculature have been found to be on the arterial side of the pulmonary circulation (Lal *et al.,* 1995), and as a result, any increase in LW would not be as a result of a hydrostatic oedema, but some other mechanism. The involvement

of non-ET_A receptor mediating changes in permeability, is supported by the findings of Kurose *et al.* (1993), who showed that ET-3-induced increases in permeability in the rat mesentery were not affected by the ET_A receptor antagonist BQ123. This would indicate that the increase in lung weight, in the isolated lung model, is due, at least in part, to a permeability change within the vasculature. However the exact mechanism responsible for the increase in lung weight remains unidentified. The big ET-1 induced increases in lung weight were attenuated by the endothelin converting enzyme inhibitor phosphoramidon, indicating that conversion of big ET-1 to ET-1 is necessary for these effects.

In summary, the experiments conducted have demonstrated the effects of a systemic hypoxia upon an isolated rat lung model, which responds with an increase in PPP and LW. This is a similar response to that seen in isolated lungs exposed to alveolar hypoxia, where the ultimate effect of hypoxia is a fall in the oxygen level within the pulmonary circulation.

The involvement of ETs has been demonstrated by the inhibition of the development of HPV and its associated increase in LW by the ET_A receptor antagonist BQ123, the ET_B receptor antagonist BQ788 and the mixed ET_A/ET_B receptor antagonist bosentan, which indicates the involvement of both the ET_A and the ET_B receptor in these responses. Furthermore, the endothelin converting enzyme inhibitor phosphoramidon, and agents which disrupted the function of the cytoskeleton and prevented secretion (colchicine and phalloidin) and inhibited peptide synthesis (cycloheximide) also prevented the increases in PPP and LW induced by hypoxia.

Exposure to hypoxia in a recirculating system results in an elevation of perfusate ET-1 levels, showing that ET-1 is released in response to hypoxia in the rat lung. However, tissue ET-1 levels do not alter significantly. The use of a peptide synthesis inhibitor (cycloheximide) and agents which disrupt the cytoskeleton and prevent secretion (colchicine and phalloidin) which prevent the increase in perfusate ET-1 levels indicates that the increase in ET-1 observed is as a result of

de novo synthesis and not as a result of release from preformed stores. Furthermore, the amount of ET-1 released into the perfusate in response to hypoxia in the lung falls within the physiological concentration demonstrated to cause an increase in PPP and LW in this model.

Prepro ET-1 mRNA levels increase in whole lung homogenate following hypoxic perfusion, suggesting either an increase in the half life of prepro ET-1 mRNA or upregulation of ET-1 production.

Taken together, these results suggest that ETs and their receptors play an important role in the response seen to hypoxia in the rat lung.

Future Work

Future studies of the involvement of endothelins in the effects of hypoxia in the rat lung include:

a) Investigation into how hypoxia turns on endothelin synthesis, and how the decrease in PO_2 is detected.
b) Investigation of the mechanism responsible for the increase in LW in response to hypoxia.
c) Measurement of ET release over time.
d) Determination of the cell type responsible for the ET release.
e) Investigation of the role of the ET receptor subtypes in the responses to hypoxia

SECTION 5

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SECTION 6

Appendix

Appendix 1

Abbreviations

A I	Angiotensin-I
A II	Angiotensin-II
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
Big ET	Big Endothelin
Big ET-1	Big Endothelin-1
CGRP	Calcitonin gene related peptide
Cys	Cysteine
DAG	Diacylglycerol
ECE	Endothelin converting enzyme
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
HPV	Hypoxic pulmonary vasoconstriction
IP3	Inositol 1,4,5-triphosphate
L-NAME	Nω-nitro-L-Arginine methyl ester
LW	Lung weight
NO	Nitric oxide
PCR	Polymerase chain reaction
PG	Prostaglandin

PGI2	Prostacyclin
PIP	Pulmonary inflation pressure
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A_2
PLC	Phosphoinositide specific phospholipase C
PLD	Phospholipase D
PO2	Partial pressure of oxygen
PPP	Pulmonary perfusion pressure
PSS	Physiological salt solution
RT	Reverse transcription
SDW	Sterile distilled water
SEM	Standard error of the mean
Sx	Sarafotoxin
Sx6C	Sarafotoxin 6C
Trp	Tryptophan
TX	Thromboxane
Val	Valine
VIC	Vasoactive intestinal contracting factor

Appendix 2

Publications

Smith, R.M., Roach, A.G., Brown, T.J., Williams, K.I. & Woodward, B. (1997). Evidence for endothelin involvement in the pulmonary vasoconstrictor response to systemic hypoxia in the isolated rat lung. *J. Pharmacol. Exp. Ther.,* **283,** 419-425.

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